University of Alberta

IDENTIFICATION AND CHARACTERIZATION OF MULTIDRUG EFFLUX PUMPS IN THE *BURKHOLDERIA CEPACIA* COMPLEX

by

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ABSTRACT

Infections caused by the *Burkholderia cepacia* complex, an opportunistic pathogen best known for infecting cystic fibrosis patients, are notoriously difficult to treat due to widespread antibiotic resistance. The objective of this study was to determine the role of multidrug efflux pumps in the overall antibiotic resistance of these organisms. In this study, six putative efflux pumps belonging to the resistance nodulation division (RND) family were identified. Several attempts were made to characterize the efflux systems by deletion and overexpression analysis. RT-PCR experiments showed that only one of the efflux systems was expressed in wild-type cells. Potential inducers of efflux pumps were tested with RT-PCR experiments. The presence of salicylate and chloramphenicol had no effect on efflux pump expression. However, one efflux system appeared to be induced during late-logarithmic growth. In addition, a Flp/FRT system was developed for the creation of markerless mutations in the *B. cepacia* complex.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
Δ	Delta (used to represent deletion)
λ	Lambda
Φ	Phi
ABC	ATP-binding cassette
BLAST	Basic local alignment search tool
BLASTP	Protein-protein basic local alignment search tool
bp	base pair
cDNA	Complementary or copy DNA
CF	Cystic fibrosis
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *Escherichia coli*
Flp	Flippase
FRT	Flp-recombinase target
HK	Sensor histidine kinase
HSL	Homoserine lactone
kb	Kilobase
LPS	Lipopolysaccharide
MATE	Multidrug and toxic compound extrusion
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
NCCLS	National Commmittee for Clinical Laboratory Standards

OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RND	Resistance nodulation cell division
RR	Response Regulator
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCP	Small cryptic plasmid
SDS	Sodium dodecyl sulfate
SMR	Small multidrug resistance
TAE	Tris-acetate EDTA

Chapter 1: Introduction

1. INTRODUCTION

1.1 The *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex refers to a group of closely related species of Gram-negative, non-spore forming bacilli. Originally known as *Pseudomonas cepacia* (Burkholder, 1950), the organism has undergone many changes in taxonomy. The genus *Burkholderia* was created in 1992 (Yabuuchi *et al.*, 1992) and later divided into five genomovars representing groups of genetically distinct, phenotypically similar isolates (Vandamme *et al.*, 1997). Once differential tests became available, the genomovars were assigned species names. There are currently nine species in the *B. cepacia* complex: *B. cepacia* (genomovar I) (Vandamme *et al.*, 1997), *B. multivorans* (genomovar II) (Vandamme *et al.*, 1997), *B. cenocepacia* (genomovar III) (Vandamme *et al.*, 1997; Vandamme *et al.*, 2003), *B. stabilis* (genomovar IV) (Vandamme *et al.*, 1997; Vandamme *et al.*, 2000), *B. vietnamiensis* (genomovar V) (Vandamme *et al.*, 1997), *B. dolosa* (genomovar VI) (Coenye *et al.*, 2001a; Vermis *et al.*, 2004), *B. ambifaria* (genomovar VII) (Coenye *et al.*, 2001b), *B. anthina* (genomovar VIII) (Vandamme *et al.*, 2002), and *B. pyrrocinia* (genomovar IX) (Vandamme *et al.*, 2002).

Since its original discovery as a plant pathogen (Burkholder, 1950), the *B. cepacia* complex has emerged as an organism of interest in the fields of agriculture, bioremediation, and medicine. Members of the *B. cepacia* complex cause sour skin disease of onions (Burkholder, 1950). This pathogenicity involves a plasmid-encoded pectate hydrolase responsible for tissue maceration (Gonzalez *et al.*, 1997) and a type IV secretion system involved in secretion of plant cytotoxic factors (Engledow *et al.*, 2004). However, in many ways, the *B. cepacia* complex is beneficial to agriculture. Members of the *B. cepacia* complex are associated with the roots of commercial crops such as maize, rice, and wheat, sometimes even colonizing internal root tissue (Gillis *et al.*, 1995; Hebbar *et al.*, 1992b; Nacamulli *et al.*, 1997; Viallard *et al.*, 1998). Interaction with the *B. cepacia* complex can enhance crop growth (Bevivino *et al.*, 1998; Clark and Parke, 1996; Germida and Walley, 1996; Tran Van *et al.*, 2000). For example, inoculation of rice with *B. vietnamiensis* increases yield by 13-22% (Tran Van *et al.*, 2000). *B. cepacia* complex strains are also useful biocontrol agents against fungal pathogens such as *Pythium* spp. (Bowers and Parke, 1993; Heungens and Parke, 2000; King and Parke, 1993; Mao *et al.*, 1998), *Rhizoctonia solani* (Homma *et al.*, 1989; Kang *et al.*, 1998), and *Fusarium* spp. (Bevivino *et al.*, 1998; Hebbar *et al.*, 1992a). The mechanism of biocontrol is often attributed to production of antifungal metabolites such as antibiotics (Burkhead *et al.*, 1994; Cartwright *et al.*, 1995; Homma *et al.*, 1989; Jiao *et al.*, 1996; Kang *et al.*, 1998; Lee *et al.*, 1994; Meyers *et al.*, 1987; Moon *et al.*, 1996; Parker *et al.*, 1984; Roitman *et al.*, 1990) and siderophores (Bevivino *et al.*, 1994; Meyer *et al.*, 1995; Sokol *et al.*, 1999). In addition to agricultural uses, the *B. cepacia* complex is a potential bioremediation agent. *B. cepacia* complex members are able to use a wide array of carbon sources, including soil and groundwater contaminants such as phthalates, herbicides and chlorinated hydrocarbons (Krumme *et al.*, 1993; Ribbons *et al.*, 1984; Sangodkar *et al.*, 1988; Shields and Reagin, 1992). Despite beneficial applications in agriculture and bioremediation, there is concern about potential harm to people susceptible to infections by the organism (Holmes *et al.*, 1998).

Although there are some examples of *B. cepacia* complex infections in immunocompetent individuals (Ledson *et al.*, 1998; Pujol *et al.*, 1992; Taplin *et al.*, 1971; Wong *et al.*, 1991), the organism primarily poses a severe danger to immunocompromised patients. Members of the *B. cepacia* complex can be recovered from people with human immunodeficiency virus (HIV) and cancer (Pegues *et al.*, 1993; Verghese *et al.*, 1994; Zinner, 1999). *B. cepacia* complex septicemia and pneumonia is the second-leading cause of death in people with chronic granulomatous disease (CGD), a disorder of the polymorphonuclear leukocytes (Johnston, 2001). Depending on the clinic, 10 to 40% of cystic fibrosis (CF) patients are colonized with the *B. cepacia* complex (Govan and Deretic, 1996). This colonization has variable outcomes. The majority of people are asymptomatic carriers or develop chronic infections. However, approximately 20% of CF patients infected with *B. cepacia* develop "*cepacia* syndrome," a severe necrotizing pneumonia and septicemia resulting in rapid clinical deterioration and early death (Isles *et al.*, 1984). Evidence of person-to-person transmission (LiPuma *et al.*, 1990) has resulted in segregation of CF patients colonized with the *B. cepacia* complex from the rest of the CF community. Although *B. cenocepacia* (genomovar III) and *B. multivorans* (genomovar II) are the most common infectious agents, members of all genomovars have been isolated from infected CF patients (Mahenthiralingam *et al.*, 2005). Members of the *B. cepacia* complex possess a vast array of virulence factors, recently reviewed by Mahenthiralingam *et al.* (2005), however, not all have a verified role in human disease. Current treatment of CF patients focuses on antibiotic therapy to decrease inflammation and bacterial numbers. However, treatment of *B. cepacia* complex infections is notoriously difficult due to widespread antibiotic resistance.

1.2 Antibiotic resistance in the *B. cepacia* complex

B. cepacia complex isolates generally have high resistance to β-lactams and aminoglycosides along with variable levels of resistance to quinolones (Burns and Saiman, 1999). Some strains of the *B. cepacia* complex are so resistant that no two-drug combination is effective against them. Therefore, triple antibiotic combinations may be needed to treat infections (Aaron *et al.*, 2000; Burns and Saiman, 1999). Clinically, antibiotic resistance of the *B. cepacia* complex is of great concern. A study of CF centres in northern Ireland found that 81% of adult patients were infected with pan-resistant genomovar III strains (Moore *et al.*, 2001). Many factors contribute to the extreme levels of resistance observed in the *B. cepacia* complex, including outer membrane impermeability, biofilm growth, β-lactamase enzymes, resistant drug targets, and multidrug efflux pumps.

Before the discovery of biofilms and efflux pumps, outer membrane impermeability was thought of as the primary drug resistance mechanism of the *B. cepacia* complex. Entry of hydrophilic compounds through water-filled porins is limited by the size and physiological properties of the solute (Hancock, 1984). Entry of hydrophobic compounds depends on diffusion across the outer membrane itself (Plesiat and Nikaido, 1992). The *B. cepacia* complex outer membrane provides a considerable barrier to the entry of hydrophilic compounds. The measured outer membrane permeability to the β-lactam antibiotic nitrocefin is 10-fold lower than that of *Escherichia coli* (Parr *et al.*, 1987). In addition, purified porin from the *B. cepacia* complex is small in size compared to *E. coli* porins (Parr *et al.*, 1987). Decreased expression of a 36-kDa outer membrane protein and no expression of a 27-kDA outer membrane protein are associated with decreased permeability and resistance to β-lactams (Aronoff, 1988). Low outer membrane permeability also contributes to aminoglycoside resistance in the *B. cepacia* complex (Moore and Hancock, 1986).

Another factor adding to the antibiotic resistance of the *B. cepacia* complex is growth in a biofilm. Biofilms are microbial communities attached to a surface and encased in a polysaccharide matrix. Growth in a biofilm is commonly associated with enhanced resistance to antimicrobial agents and the host immune system. Biofilm resistance is most likely due to a combination of different mechanisms that have been reviewed recently (Drenkard, 2003). Possibilities include: reduced penetration of drugs through the exopolysaccharide matrix, local differences in metabolic activity of cells, physiological changes caused by the activation of genetic systems, the presence of persister cells, activation of the general stress response, involvement of quorum sensing systems, and the activation of multidrug efflux pumps (Drenkard, 2003). Evidence suggests that efflux pumps do not play a significant role in biofilm resistance. Genes encoding resistance nodulation cell division (RND) efflux systems are not induced during microarray analysis of mature *Pseudomonas aeruginosa* biofilms (Whiteley *et al.*, 2001). Overexpression of MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY does not contribute significantly to increased drug resistance in *P. aeruginosa* biofilms (De Kievit *et al.*, 2001). However, genome analysis reveals the existence of 12 possible RND systems in *P. aeruginosa* (Stover *et al.*, 2000), so the involvement of other efflux systems in biofilm resistance cannot be ruled out.

Members of the *B. cepacia* complex are capable of biofilm growth *in vitro* (Buhler *et al.*, 1998; Lo *et al.*, 1996; Nivens *et al.*, 1993; Tomlin *et al.*, 2001). There is some evidence to suggest that *B. cepacia* complex biofilm formation is controlled by the *cepIR* quorum sensing system (Huber *et al.*, 2001; Huber *et al.*, 2002). However, under some growth conditions, biofilm formation is independent of quorum sensing (Conway *et al.*, 2002). Although it has not been established what form the *B. cepacia* complex is found in during infections of the CF lung, some interesting correlations exist between biofilm growth and pathogenesis. Infection with the *B. cepacia* complex usually occurs in CF patients who are already colonized with *P. aeruginosa* (Govan and Deretic, 1996). *In vitro* evidence of mixed biofilm growth and interspecies communication with *P. aeruginosa* raises the possibility that they could co-regulate virulence factors in the CF lung (Lewenza *et al.*, 2002; Riedel *et al.*, 2001). Genomovar II and III *B. cepacia* complex isolates, which are the most invasive (Cieri *et al.*, 2002; Keig *et al.*, 2002) and most frequently isolated from CF patients (Mahenthiralingam *et al.*, 2005), also form the most abundant biofilms *in vitro* (Conway *et al.*, 2002). If these isolates do form biofilms in the CF lung, it could contribute to antibiotic resistance since *B. cepacia* biofilm cells are 15 times less susceptible to ceftazidime and ciprofloxacin than planktonic bacteria at an equivalent stage of growth (Desai *et al.*, 1998).

Other mechanisms of resistance in the *B. cepacia* complex include the ability to degrade certain antibiotics. For example, members of the *B. cepacia* complex are able to use penicillin G as a sole carbon source (Beckman and Lessie, 1979). Some strains, including CF isolates, have inducible β-lactamases (Chiesa *et al.*, 1986). For example, *B. multivorans* strain 249, has two inducible β-lactamases: a cephalosporinase (Beckman and Lessie, 1980) and a penicillinase (Prince *et al.*, 1988). The *penA* and *penR* genes, encoding the penicillinase and its regulator respectively, have been cloned and sequenced. Expression of *penA* in *E. coli* confers resistance to benzylpenicillin, carbenicillin, and cefuroxime, but not imipenem (Trepanier *et al.*, 1997).

Resistance of the *B. cepacia* complex to some antimicrobials is the result of resistant drug targets. At least some trimethoprim resistance is explained by production of a trimethoprim-resistant dihydrofolate reductase (Burns *et al.*, 1989b). Specific penicillin-binding proteins (PBP) are less susceptible to the actions of β-lactam antibiotics (Mahenthiralingam *et al.*, 2005). The specific structure of *B. cepacia* complex LPS confers resistance to cationic antimicrobial peptides and polymyxin. *B. cepacia* complex LPS contains D-glycero-D-talo-2-octulosonic acid (KO) and 4-amino-4-deoxy-L-arabinose (Ara4N) (Isshiki *et al.*, 1998), both of which are expected to reduce the binding affinity of LPS for cationic antibiotics. However, although *B. cepacia* complex LPS is somewhat resistant to the actions of polymyxin B, the LPS does bind polymyxin B with high affinity (Shimomura *et al.*, 2003). In addition to the mechanisms of resistance discussed above, active efflux systems are also involved in *B. cepacia* complex antimicrobial resistance (Burns *et al.*, 1996; Fehlner-Gardiner and Valvano, 2002; Wigfield *et al.*, 2002).

1.3 Multidrug efflux pumps

1.3.1 Classes of bacterial drug efflux pumps

Antibacterial efflux pumps fall into five larger classes of transporters. Members of the ATP-binding cassette (ABC) superfamily use ATP-hydrolysis to drive transport of sugars, amino acids, ions, drugs, iron complexes, polysaccharides, and proteins (Fath and Kolter, 1993). ABC pumps consist of two subunits: an integral membrane protein with six transmembrane helices and an ATP-binding subunit located at the cytoplasmic face of the membrane. The two proteins may be non-covalently associated or linked in a single polypeptide chain. The complete system usually functions as a dimer (Fath and Kolter, 1993). Although ABC-type drug efflux pumps are not common in bacteria (Li and Nikaido, 2004), some examples include *Lactococcus lactis* multidrug pump LmrA (van Veen *et al.*, 1996) and *Escherichia coli* drug-specific pump MacAB (Kobayashi *et al.*, 2001b).

The major facilitator superfamily (MFS) pumps are driven by electro-chemical gradients, usually proton-motive force, in the transport of sugars, metabolites, anions, and drugs (Saier *et al.*, 1998). Two families within this group are involved with drug efflux. Pumps with 12 transmembrane helices have a large cytoplasmic loop between helices six and seven. Pumps with 14 transmembrane domains have a smaller cytoplasmic loop. MFS systems function as either dimers or trimers (Saier *et al.*, 1998). A well characterized example of an MFS pump is EmrAB of *E. coli* (Lomovskaya and Lewis, 1992).

The multidrug and toxic compound extrusion (MATE) family is a newly discovered class of efflux pumps. Members of this family have 12 transmembrane domains with a similar topology, but not homology, to MFS efflux pumps (Brown *et al.*, 1999). Two known bacterial multidrug efflux pumps of this class are NorM of *Vibrio parahaemolyticus* and YdhE of *E. coli*, both conferring resistance to cationic dyes, aminoglycosides, and fluoroquinolones (Morita *et al.*, 1998).

The small multidrug resistance (SMR) family of efflux pumps contains drug:proton antiporters. The protein is small, approximately 110 amino acids, and contains four transmembrane domains (Arkin *et al.*, 1996). SMR pumps likely function as trimers (Yerushalmi *et al.*, 1996). Two well known SMR pumps are *Staphylococcus aureus* Smr and *E. coli* EmrE. Although these are considered multidrug pumps, their substrate ranges are limited to lipophilic cations such as antiseptics and disinfectants (Grinius *et al.*, 1992; Schuldiner *et al.*, 2001).

The resistance-nodulation-cell division (RND) family was named after the functions of its original members: transporters involved in metal resistance, proteins required for nodulation of alfalfa roots by *Rhizobium meliloti*, and EnvD (now known as multidrug transporter AcrF) which was originally thought to be involved in *E. coli* cell division (Saier *et al.*, 1994). RND transporters have a unique topology with 12 transmembrane segments and two large periplasmic loops. The efflux system functions as a trimer (Poole, 2004). All known RND drug pumps are multi-drug transporters. Proton motive force drives efflux of a broad range of structurally unrelated compounds including most classes of antibiotics, biocides, dyes, detergents, metabolic inhibitors, aromatic hydrocarbons, cationic antimicrobial peptides, toxic fatty acids, bile salts, and quorum sensing signals (Poole, 2004). As such, they play a major role in the resistance of Gram-negative organisms to a variety of clinically relevant antimicrobials. Numerous examples exist in Gram-negative organisms (Table 1.1).

1.3.2 Tripartite structure of Gram-negative efflux pumps

Drug efflux pumps in Gram-negative organisms face the challenge of transporting the substrate across the outer membrane. Such pumps often work in complex with an outer membrane protein and a membrane fusion protein (Saier *et al.*, 1998). The best

Table 1.1 A selection of resistance nodulation cell division (RND) family multidrug efflux pumps from Gram-negative bacteria

Organism	**Efflux system component**			**Regulator**	**Substrates**	**References**
	MFP	**IMT**	**OMP**			
Burkholderia cenocepacia	CeoA	CeoB	OpcM	CeoR	CM, CP, TP	(Burns *et al.*, 1996; Nair *et al.*, 2004)
Escherichia coli	AcrA	AcrB	TolC	AcrR, MarA, SoxS, Rob, SdiA	AC, AH, BA, BL, BS, CM, CV, EB, ER, FA, FU, FQ, LN, MX, NV, PO, RF, SDS,TC, TS, TX	(Fralick, 1996; Jellen-Ritter and Kern, 2001; Kopytek *et al.*, 2000; Ma *et al.*, 1993; O'Neill *et al.*, 2002; Tsukagoshi and Aono, 2000)
	AcrA	AcrD	TolC	SdiA, BaeR, CpxR, OmpR	AG, BL, DC, FU, NV, SDS	(Elkins and Nikaido, 2002; Hirakawa *et al.*, 2003; Rosenberg *et al.*, 2000)
	AcrE	AcrF	TolC	AcrS, SdiA	AC, AH, BL, BS, FQ, LN, ML, RD, SDS, TC, TP	(Jellen-Ritter and Kern, 2001; Kobayashi *et al.*, 2001a; Ma *et al.*, 1994; Nishino and Yamaguchi, 2001b)
	MdtA (YegM)	MdtBC (YegNO)	TolC	BaeSR	BL, BS, DC, NV, SDS	(Baranova and Nikaido, 2002; Nagakubo *et al.*, 2002; Nishino and Yamaguchi, 2001b)
	YhiU	YhiV	TolC	EvgAS	BA, BL, BS, CV, DC, DOX, EB, ER, RD, SDS	(Masuda and Church, 2002; Nishino and Yamaguchi, 2001b, 2002; Sulavik *et al.*, 2001)
Neisseria gonorrhoeae	MtrC	MtrD	MtrE	MtrR, MtrA	AZ, CV, EB, FA, ML, PN, RF, TX	(Hagman *et al.*, 1995; Hagman *et al.*, 1997; Pan and Spratt, 1994; Veal *et al.*, 1998; Zarantonelli *et al.*, 1999)
Pseudomonas aeruginosa	MexA	MexB	OprM	MexR, NalC (PA3721)	AC, AG, AH, BL, CL, CM, CV, EB, FQ, HL, IR, ML, NV, RD, SDS, SM, TC, TL, TG, TP, TPP, TS	(Dean *et al.*, 2003; Li *et al.*, 1995; Masuda *et al.*, 2000a; Okamoto *et al.*, 2002a, b; Poole *et al.*, 1993a; Poole *et al.*, 1996b)
	MexC	MexD	OprJ	NfxB	AC, AH, BL, CL, CM, CP, EB, FQ, NV, RD, SDS, SM, TC, TG, TP, TPP, TS	(Masuda *et al.*, 2000a; Okamoto *et al.*, 2002a, b; Poole *et al.*, 1996a)
	MexE	MexF	OprN	MexT	AH, CM, FQ, TP, TS	(Kohler *et al.*, 1997)
	MexX	MexY	OprM	MexZ	AG, BL, ER, FQ, TC, TG	(Aires *et al.*, 1999; Masuda *et al.*, 2000b; Mine *et al.*, 1999; Okamoto *et al.*, 2002a; Westbrock-Wadman *et al.*, 1999)
	MexH	MexI	OpmD	PA4203?	VA, EB, NV, RD, AC	(Aendekerk *et al.*, 2002; Sekiya *et al.*, 2003)
	MexJ	MexK	OprM	MexL	AC, CM, EB, ER, FQ, TC, TS	(Chuanchuen *et al.*, 2002; Li *et al.*, 2003)

AC = acriflavine; **AG** = aminogylcosides; **AH** = aromatic hydrocarbons; **AZ** = azithromycin; **BA** = benzalkonium; **BL** = β-lactams; **BS** = bile salts; **CL** = cerulenin; **CM** = chloramphenicol; **CP** = ciprofloxacin; **CV** = crystal violet; **DC** = deoxycholate; **DOX** = doxyrubicin; **EB** = ethidium bromide; **ER** = erythromycin; **FA** = fatty acids; **FU** = fusidic acid; **FQ** = fluoroquinolones; **HL** = homoserine lactones; **IMT** = inner membrane transporter; **IR** = irgasan; **LN** = linezolid; **MFP** = membrane fusion protein; **ML** = macrolides; **MX** = methotrexate; **NV** = novobiocin; **OMP** = outer membrane protein; **PN** = penicillin; **PO** = pine oil; **RD** = rhodamine; **RF** = rifampicin; **SDS** = sodium dodecyl sulphate; **SM** = sulphonamides; **TC** = tetracycline; **TG** = tigecycline; **TP** = trimethoprim; **TPP** = tetraphenyl phosphonium; **TS** = triclosan; **TX** = Triton X-100; **VA** = vanadium. In instances where only one member of an antimicrobial class has been tested, that member is identified. Where several members of an antimicrobial class are known to be substrates, the class is identified.

studied examples of tripartite efflux structures are *E. coli* AcrAB-TolC and *P. aeruginosa* MexAB-OprM, both members of the RND superfamily.

Inner membrane transporter. The inner membrane transporter is responsible for binding substrates and energizing active transport out of the cell. The crystal structure of *E. coli* transporter AcrB has been determined at high resolution (Figure 1.1) (Murakami *et al.*, 2002). The structure revealed that AcrB functions as a trimer. The trimer contains two domains, a transmembrane region and a periplasmic headpiece. The transmembrane domain consists of the 12 transmembrane helices of each monomer, arranged in a ring with a central hole. The hole is large and likely filled with phospholipids of the cytoplasmic membrane (Murakami *et al.*, 2002). The periplasmic headpiece contains three vestibules that are open to the periplasm, forming channels into a central cavity inside the pump. The cavity contains numerous aromatic, polar, and charged residues possibly involved in substrate interaction. Located above the central cavity is a closed pore formed by three helices (Murakami *et al.*, 2002). Conformational change of this pore is important since mutations fixing the pore in a closed state result in almost complete loss of transport (Murakami *et al.*, 2004). The top of the periplasmic headpiece resembles a funnel. The diameter of the funnel is approximately equal to the diameter of TolC, suggesting that it could act as a docking-site for the outer membrane protein (Murakami *et al.*, 2002).

The crystal structure of AcrB also suggests a possible proton translocation pathway. Three charged residues in the middle of transmembrane helices four and ten form ion pairs (Murakami *et al.*, 2002). The amino acids involved in these pairs are highly conserved and essential for the function of several RND pumps including MexB

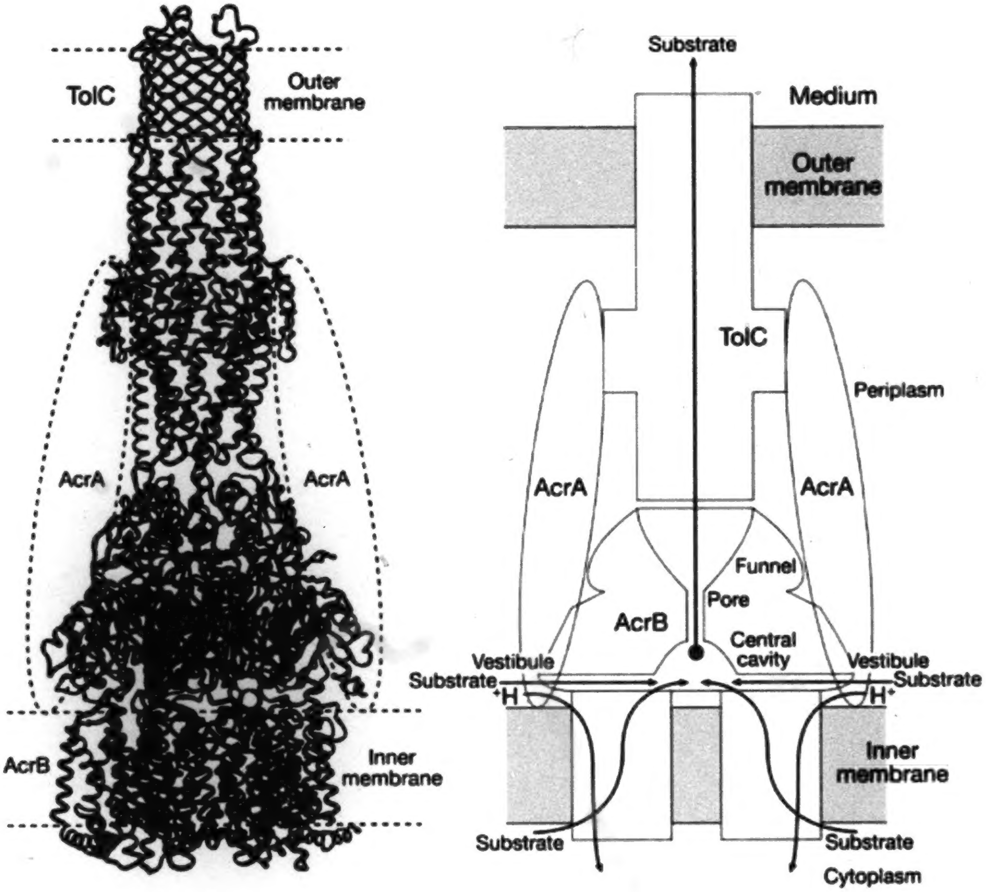
TolC
Outer membrane
AcrA
AcrA
AcrB
Inner membrane
Substrate
Medium
Outer membrane
TolC
Periplasm
AcrA
AcrA
Funnel
Pore
AcrB
Central cavity
Vestibule
Vestibule
Substrate
Substrate
+H
H+
Inner membrane
Substrate
Substrate
Cytoplasm

Figure 1.1 Model of the AcrAB-TolC complex. Taken directly from Murakami *et al.*, 2002. The inner membrane transporter, AcrB, is responsible for proton translocation and substrate binding. The outer membrane protein TolC, shown docked into the AcrB structure, allows transport across the outer membrane.

(Guan and Nakae, 2001) and MexF (Aires *et al.*, 2002) of *P. aeruginosa* as well as CzcA of *Ralstonia* spp. (Goldberg *et al.*, 1999).

In addition to proton translocation, the inner membrane transporter is responsible for binding efflux substrates. The majority of evidence suggests that substrates are captured in the periplasm (Li and Nikaido, 2004). However, recent evidence suggests that some RND pumps are capable of both cytoplasmic and periplasmic capture. Reconstituted *E. coli* efflux pump AcrD is able to transport aminoglycosides from the outside (representing the cytoplasm) and inside (representing the periplasm) of proteoliposomes (Aires and Nikaido, 2005). A great deal of research has focused on which components of RND transporters are involved in substrate binding. The importance of the periplasmic loops in substrate recognition has been well established by the construction of chimeric transporters (Eda *et al.*, 2003; Elkins and Nikaido, 2002; Tikhonova *et al.*, 2002) and mutagenesis experiments (Franke *et al.*, 2003; Mao *et al.*, 2002; Middlemiss and Poole, 2004). However, X-ray crystallography of AcrB in the presence of structurally diverse ligands also provides convincing evidence that the transmembrane domain is responsible for substrate specificity (Yu *et al.*, 2003). Three molecules of ligand bind simultaneously in the central cavity via hydrophobic, aromatic stacking, and van der Waals interactions. Slightly different subsets of residues are involved in binding different ligands. The binding sites are near the proposed location of the cytoplasmic membrane within the central cavity. Surprisingly, most residues identified as drug binding sites are located in the transmembrane domain, not the periplasmic loops. However, if drugs use the periplasmic vestibules to enter the central cavity, the periplasmic domains could still limit which substrates have access to the binding sites (Yu *et al.*, 2003).

Outer membrane protein. The outer membrane protein facilitates drug efflux across the outer membrane of Gram-negative organisms. The crystal structure of well-characterized outer membrane protein TolC of *E. coli* has been obtained (Figure 1.1) (Koronakis *et al.*, 2000). TolC functions as a trimer with two major domains. A β-barrel is embedded in the outer membrane and opens to the extracellular space. The trimer also has an α-helical cylinder that extends into the periplasm, gradually narrowing until it tapers to a close. The cylinder has a large interior cavity, a likely route for substrate passage. The bottom of the cylinder is sealed by a set of coiled helices, suggesting that these helices must open in order for substrates to pass (Koronakis *et al.*, 2000). Later, the crystal structure of another RND efflux pump outer membrane protein, *P. aeruginosa* OprM, was also solved (Akama *et al.*, 2004a). The structure is very similar to that of TolC, with some notable differences. The diameter of the outer membrane β-barrel was smaller than its *E. coli* counterpart and is restricted by the presence of three short loops protruding into the centre of the pore. In order for substrates to pass through OprM, the top β-barrel pore must open as well as the closed tip of the periplasmic cylinder (Akama *et al.*, 2004a).

Support for the necessity of a conformational change in outer membrane proteins comes from reconstitution experiments. *P. aeruginosa* OprM reconstituted in liposome membranes has some channel-forming abilities, allowing diffusion of amino acids and peptides but not antibiotics (Yoshihara *et al.*, 2002). Weak pore-forming activity results from reconstitution of TolC and OprM lipid bilayers (Benz *et al.*, 1993; Wong and

Hancock, 2000). Together, these results are consistent with the idea that outer membrane proteins are gated-channels that must be opened to allow drug efflux. The TonB protein is involved in the opening of many gated outer membrane receptors (Postle, 1993). Recently, it has been suggested that TonB may also play a role in active efflux. TonB is required for full efflux activity of *P. aeruginosa* multidrug efflux pump MexAB-OprM (Zhao *et al.*, 1998). Similarly, TonB is involved in efflux systems in *Pseudomonas putida* (Godoy *et al.*, 2001) and *Neisseria gonorrhoeae* (Rouquette-Loughlin *et al.*, 2002). However, TonB is not required for the function of *E. coli* AcrAB-TolC (Li and Nikaido, 2004).

Membrane fusion protein. The third member of the tripartite complex is the membrane fusion protein, also known as the periplasmic linker protein. Recently, the crystal structure of *P. aeruginosa* MexA, a well-characterized membrane fusion protein, was determined by two research groups (Akama *et al.*, 2004b; Higgins *et al.*, 2004). The MexA monomer is an elongated structure with three domains. First, a β-barrel domain is formed by β-strands and an α-helix and bears an overall resemblance to ligand binding domains. Second, a lipoyl/biotinyl domain is conserved in membrane fusion proteins. Third, there is an α-helical hairpin that contains both N-terminal and C-terminal helices. Both groups also note a fourth, disordered domain that was not resolved by the crystal structure. This domain contains the N-terminal and C-terminal residues of MexA (Akama *et al.*, 2004b; Higgins *et al.*, 2004).

The MexA crystal contained two twisted funnel shapes (a 7-mer and a 6-mer) united to form a 13-mer cylinder with an internal space and open ends. However, this structure is unlikely to be formed *in vivo*. Both research groups used an altered form of

MexA lacking the N-terminal fatty acyl moiety (Akama *et al.*, 2004b; Higgins *et al.*, 2004). Although the altered protein is fully functional in regards to antibiotic efflux, it no longer attaches to the inner membrane (Yoneyama *et al.*, 2000). *In vivo*, the attachment of MexA to the inner membrane would prevent head-to-head arrangement of the 7-mer and 6-mer. Based on the crystal structure, three suggestions have been made regarding the true form of MexA multimers. One possibility is that nine molecules of MexA could form a ring attached to the inner membrane and surrounding the other efflux components (Higgins *et al.*, 2004). A second suggestion states that 12 MexA molecules could wind around the MexB-OprM interaction site (Akama *et al.*, 2004b). Another possibility is that three MexA dimers could interact with the periplasmic end of OprM by means of α-helices. The C-terminal region could then interact with MexB (Akama *et al.*, 2004b). There is still no conclusive evidence for the actual *in vivo* state of MexA. Quantification of MexA, MexB, and OprM by immunoblotting, suggests that there are six molecules of MexA for every trimer of MexB and OprM (Narita *et al.*, 2003). Chemical cross-linking studies of membrane fusion proteins AcrA (Zgurskaya and Nikaido, 2000) and HlyD (Thanabalu *et al.*, 1998) support a trimeric form. Low resolution crystallography of AcrA shows the appearance of two identical rings, taken to be an AcrA dimer (Avila-Sakar *et al.*, 2001).

Another question remains about the function of membrane fusion proteins. Numerous studies have shown they are essential for efflux in intact cells (Aires and Nikaido, 2005; Elkins and Nikaido, 2002; Li *et al.*, 1995; Ma *et al.*, 1995; Poole *et al.*, 1993a). Suggestions include bringing the inner and outer membranes together, assembly and stabilization of the tripartite complex, activation of efflux, and drug-binding.

In the membrane fusion model, the membrane fusion protein brings the inner and outer membranes into close contact, facilitating substrate transfer between the inner membrane transporter and the outer membrane protein. Evidence for this model is mostly circumstantial. Sequence similarity to a viral membrane fusion protein and the presence of two conserved hydrophobic regions near the N and C terminal ends of periplasmic adaptors suggest that terminal ends could interact with the membranes (Dinh *et al.*, 1994). Furthermore, membrane fusion protein AcrA is attached to the inner membrane via an N-terminal lipid moiety (Ma *et al.*, 1993) and the AcrA monomer exists as an elongated shape that could span the periplasm (Zgurskaya and Nikaido, 1999a). Structural predictions reveal the presence of two coiled-coils separated by a gap and flanked on each side by two copies of a lipoyl motif. This structure suggests a mechanism for membrane fusion whereby this region could fold back on itself to form an α-helical hairpin (Johnson and Church, 1999). Direct evidence for the membrane fusion model comes from the observation that AcrA alone causes transfer of lipids between two vesicle populations without mixing the aqueous contents (Zgurskaya and Nikaido, 1999b). However, recent studies strongly suggest that the role of membrane fusion proteins is not to bring together the inner and outer membranes. The crystal structure of MexA demonstrates that the N- and C-terminal domains are in close contact with each other, precluding the attachment of one terminus to each membrane (Akama *et al.*, 2004b; Higgins *et al.*, 2004). Also, evidence that the C-terminus of AcrA contacts the inner membrane transporter excludes it from interaction with the outer membrane (Elkins and Nikaido, 2003; Touze *et al.*, 2004).

Other evidence suggests that membrane fusion proteins play an important role in assembly of the Gram-negative tripartite efflux complex. Numerous *in vivo* cross-linking studies show interaction of membrane fusion proteins HlyD (Thanabalu *et al.*, 1998), AcrA (Touze *et al.*, 2004; Zgurskaya and Nikaido, 2000), and MexA (Nehme *et al.*, 2004) with their corresponding transporters. This interaction is fairly specific, since AcrA functions with non-cognate transporters AcrD (Elkins and Nikaido, 2002), AcrF (Kobayashi *et al.*, 2001b), and YhiV but not MdtBC (Elkins and Nikaido, 2003). Similarly, membrane fusion proteins AcrA (Husain *et al.*, 2004; Touze *et al.*, 2004) and MexA (Mokhonov *et al.*, 2004) interact with outer membrane proteins in the absence of the inner membrane transporter. Additional support for an interaction between membrane fusion proteins and outer membrane proteins is that most extragenic suppressors of *tolC* mutants are found in *acrA* (Gerken and Misra, 2004). Furthermore, most evidence suggests that inner membrane transporters and outer membrane proteins are unable to interact independently. Without the membrane fusion protein MexA, MexB and OprM do not co-purify in a pull-down assay (Mokhonov *et al.*, 2004). Although AcrB and TolC can be chemically cross-linked independently of AcrA, isothermal titration calorimetry (ITC) suggests that there is no stable interaction between AcrB and TolC. Thus, cross-linking results merely represent close physical proximity within the cell (Touze *et al.*, 2004). Attempts to construct chimeric efflux pumps confirms the importance of membrane fusion proteins (Mokhonov *et al.*, 2004; Yoneyama *et al.*, 1998). The above evidence suggests that membrane fusion proteins are essential for stable assembly of the tripartite complex.

In addition to a role in assembly of the tripartite complex, a variety of more active roles have been proposed for the membrane fusion protein. The periplasmic end of outer membrane protein TolC is sealed by sets of coiled helices. In order for efflux to occur, TolC must transition into an open state (Koronakis *et al.*, 2000). It is possible that such a transition could be triggered by protein-protein interaction between TolC and the membrane fusion protein (Andersen *et al.*, 2002). Membrane fusion proteins may also activate inner membrane transporters. RND transporter AcrD reconstituted in proteoliposomes is unable to transport aminoglycosides without membrane fusion protein AcrA (Aires and Nikaido, 2005). Previously, AcrA was shown to stimulate AcrB activity. At the time, this was attributed to AcrA bringing the vesicles into close contact (Zgurskaya and Nikaido, 1999b). However, this result can be reinterpreted as AcrA activating efflux by AcrB. Interestingly, AcrA undergoes pH-induced oligomerization and conformational change *in vitro*, providing a link to the proton-gradient driven activity of AcrB (Ip *et al.*, 2003). Although AcrA seems to stimulate efflux activity, the same may not be true for all membrane fusion proteins. Reconstituted CzcA, an RND transporter from *Ralstonia* sp., has considerable activity in the absence of its cognate membrane fusion protein (Goldberg *et al.*, 1999). Another possible role for membrane fusion proteins is suggested by direct involvement of EmrA (Borges-Walmsley *et al.*, 2003) and HlyD (Balakrishnan *et al.*, 2001; Thanabalu *et al.*, 1998) in substrate binding. Although the importance of membrane fusion proteins in efflux systems is well-established, their *in vivo* state and function remain a mystery.

1.3.3 Regulation of drug efflux pumps

The expression of many drug efflux pumps is subject to elaborate regulatory control. The wide range of molecules that can be transported by some multidrug pumps raises the possibility that normal metabolites may be extruded. One study demonstrated that mutations in the adenine, glutamate, methionine, and cysteine biosynthetic pathways cause an increase in transcription of the *acrAB* efflux system and a corresponding increase in resistance to nalidixic acid (Helling *et al.*, 2002). This could explain the finding that overexpression of efflux pumps AcrAB (Ma *et al.*, 1993) and TetA (Eckert and Beck, 1989; Hickman *et al.*, 1990) can be toxic for cells. Similar difficulties are found for the *E. coli* AcrEF (Klein *et al.*, 1991) and *Neisseria gonorrhoeae* MtrCD (Pan and Spratt, 1994) efflux systems. Overexpression of multidrug efflux pumps in *P. aeruginosa* is associated with decreased virulence and decreased survival, emphasizing the importance of efflux pump regulation (Kohler *et al.*, 2001; Linares *et al.*, 2005; Sanchez *et al.*, 2002a). The majority of bacterial drug efflux pumps are controlled by transcriptional regulatory proteins. Regulation can occur at the global or local level by repressors, activators, or two component systems.

Expression of *E. coli* efflux operon *acrAB* responds to at least four global regulators. The *E. coli mar* (multiple antibiotic resistance) locus consists of two units expressed divergently from operator *marO*: the *marC* gene and the *marRAB* operon. The functions of MarB and MarC remain unknown (Alekshun and Levy, 1997). MarA belongs to the XylS/AraC family of transcription activators and is known to control the expression of more than 60 chromosomal genes (Barbosa and Levy, 2000). Among these targets is efflux operon *acrAB*. MarA activates transcription from the *acrAB* operon, increasing drug efflux (Martin *et al.*, 1996). Expression of *marRAB* is regulated in turn by the repressor protein MarR (Alekshun and Levy, 1997). Induction of *marRAB* responds to a number of antibiotics, weak aromatic acids, uncoupling agents, and redox-cycling compounds, presumably by alleviating MarR repression of the operon (Ariza *et al.*, 1994; Cohen *et al.*, 1993; Seoane and Levy, 1995). However, direct binding to MarR has only been demonstrated for one inducer, salicylate (Alekshun *et al.*, 2001; Martin and Rosner, 1995).

MarA homologues SoxS (Ma *et al.*, 1996; McMurry *et al.*, 1998; White *et al.*, 1997) and Rob (Ariza *et al.*, 1995; Nakajima *et al.*, 1995; White *et al.*, 1997) also increase transcription of *acrAB*. Increases in SoxS occur when its repressor, SoxR, is inactivated in response to superoxides (Hidalgo *et al.*, 1997). Activation of Rob occurs via the binding of inducers such as dipyridyl (Jair *et al.*, 1996), fatty acids, and bile salts (Rosenberg *et al.*, 2003). Although overexpression of MarA, SoxS, and Rob each increase *acrAB* transcription, none of these regulators are responsible for *acrAB* induction in response to general stress conditions (Ma *et al.*, 1996). Therefore, some other regulatory factors must be involved. The recent finding that expression of *acrAB* is higher in slow-growing cells provides a link between quorum sensing and efflux pump regulation (Rand *et al.*, 2002). Additionally, the protein SdiA, which has homology to receptors of acyl homoserine lactone quorum sensing molecules, positively regulates *acrAB* (Rahmati *et al.*, 2002).

Local regulation is also involved in expression of the *acrAB* operon. The gene encoding a TetR family repressor protein, AcrR, is divergently transcribed from *acrAB* (Ma *et al.*, 1995; Pan and Spratt, 1994). AcrR binds to and represses expression from the *acrR* and *acrAB* promoters (Ma *et al.*, 1996). However, AcrR serves only to fine tune expression of *acrAB* because general stress conditions increase *acrAB* expression in the absence of functional AcrR. Also, general stress conditions increase transcription of *acrR* as well as *acrAB* (Ma *et al.*, 1996).

Local regulators play an important role in regulation of *P. aeruginosa* efflux operons. Each of the RND multidrug efflux systems is linked to a regulatory gene encoding a repressor or activator. MexR, a repressor belonging to the MarR family, is encoded by a gene upstream and divergently transcribed from efflux operon *mexAB-oprM*. Although *mexAB-oprM* is constitutively expressed in wild-type cells (Li *et al.*, 1995; Yoneda *et al.*, 2005), inactivation of *mexR* results in overexpression of *mexAB-oprM* and a corresponding increase in resistance to several antimicrobial agents (Adewoye *et al.*, 2002; Poole *et al.*, 1996b; Saito *et al.*, 1999; Srikumar *et al.*, 2000; Ziha-Zarifi *et al.*, 1999) as well as organic solvents (Li and Poole, 1999). MexR negatively autoregulates its own expression (Poole *et al.*, 1996b) and binds directly to the overlapping *mexR* and *mexAB-oprM* promoters (Evans *et al.*, 2001). The natural conditions that alleviate MexR repression are unknown. Unlike its homologue MarR, MexR does not interact with salicylate (Lim *et al.*, 2002). Expression of *oprM* is induced by the antibiotic imipenem, but only in one strain of *P. aeruginosa* (Kolayli *et al.*, 2004). The crystal structure of MexR suggests that the unknown ligand could be a peptide signaling molecule (Lim *et al.*, 2002).

MexR is not the only regulator of *mexAB-oprM* expression. Growth phase also regulates *mexAB-oprM* expression, with levels increasing in late logarithmic and stationary phase (Evans and Poole, 1999; Maseda *et al.*, 2004; Sanchez *et al.*, 2002b). Growth phase regulation does not require *mexR* (Evans and Poole, 1999; Sawada *et al.*, 2004), nor the *las* quorum sensing cascade (Evans and Poole, 1999; Maseda *et al.*, 2004). However, the *rhl* quorum sensing system is likely involved in growth phase regulation since autoinducer C4-HSL enhances expression of *mexAB-oprM* (Sawada *et al.*, 2004). Surprisingly, MexT, an activator of the *mexEF-oprN* efflux operon, represses C4-HSL-mediated regulation of *mexAB-oprM* (Sawada *et al.*, 2004). MexT-mediated regulation of *mexAB-oprM* expression occurs directly, through the regulatory function of MexT, and indirectly, through the action of the MexEF-OprN efflux pump which is activated by MexT (Maseda *et al.*, 2004). Numerous studies show that some mutations altering *mexAB-oprM* transcription are located outside of the *mexR* gene and intergenic region, suggesting the presence of another regulatory gene, *nalC* (Llanes *et al.*, 2004; Pumbwe and Piddock, 2000; Srikumar *et al.*, 2000; Ziha-Zarifi *et al.*, 1999). Recent studies indicate that mutations in a putative TetR family repressor are involved in the *nalC* phenotype (Cao *et al.*, 2002; Llanes *et al.*, 2004). Still, some strains of *P. aeruginosa*, *nalD*-type mutants, overexpress *mexAB-oprM* although they contain no mutations in any known regulatory genes (Llanes *et al.*, 2004).

Unlike MexAB-OprM, *P. aeruginosa* efflux pump MexCD-OprJ is not expressed in wild-type cells under normal growth conditions (Morita *et al.*, 2003; Poole *et al.*, 1996a). Expression of this pump is regulated by the NfxB repressor, encoded by a gene upstream of the *mexCD-oprJ* operon. NfxB is similar to proteins in the LacI-GalR repressor family (Poole *et al.*, 1996a). In addition to repressing *mexCD-oprJ* transcription, NfxB also negatively autoregulates its own expression (Shiba *et al.*, 1995). Using mutants possessing only one of the four major *P. aeruginosa* RND pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY, Morita *et al.* demonstrated that *mexCD-oprJ* expression is inducible. Some MexCD-OprJ substrates, tetraphenylphosphonium, ethidium bromide, rhodamine 6G, acriflavine, and disinfectants, induce expression of the pump. Other substrates of MexCD-OprJ, such as norfloxacin, erythromycin, tetracycline, chloramphenicol, streptomycin, and carbenicillin, do not induce expression (Morita *et al.*, 2001b; Morita *et al.*, 2003).

Regulation of the *mexXY* operon of *P. aeruginosa* also occurs via a repressor protein. The *mexZ* gene, encoding a putative TetR family repressor, is upstream and divergently transcribed from the *mexXY* operon (Aires *et al.*, 1999; Westbrock-Wadman *et al.*, 1999). MexZ down-regulates *mexXY* expression, because transformation of wild-type *P. aeruginosa* with *mexZXY* has no effect on resistance, but transformation with only *mexXY* substantially increases resistance (Aires *et al.*, 1999). In some cases, overexpression of *mexXY* has been observed in strains with mutations in the *mexZ* gene (Islam *et al.*, 2004; Llanes *et al.*, 2004; Vogne *et al.*, 2004). MexZ regulates *mexXY* directly, since purified MexZ protein binds the *mexZ-mexX* intergenic region (Matsuo *et al.*, 2004). Expression of *mexXY* is thought to be constitutive in wild-type cells, as mutations disrupting *mexX* result in hypersusceptibility to aminoglycosides, erythromycin, and tetracycline (Aires *et al.*, 1999). However, expression of *mexXY* was not found in wild-type cells using either real-time PCR or Western immunoblot analysis (Vogne *et al.*, 2004; Yoneda *et al.*, 2005). When cells are first exposed to sub-inhibitory levels of tetracycline, erythromycin, or gentamicin, MexX is observed in immunoblotting experiments (Masuda *et al.*, 2000b). Therefore, these three efflux pump substrates are inducers of *mexXY* expression (Masuda *et al.*, 2000b). These inducers do not appear to act via MexZ, since the presence of substrate antibiotics does not change the DNA-binding ability of MexZ (Matsuo *et al.*, 2004). Some antibiotic resistant, *mexXY*-overexpressing strains of *P. aeruginosa* have wild-type *mexZ* genes (Islam *et al.*, 2004; Llanes *et al.*, 2004; Vogne *et al.*, 2004). Therefore, it seems likely that an unidentified locus plays a role in regulating *mexXY* expression.

In contrast to other negatively controlled *P. aeruginosa* RND pumps, expression of *mexEF-oprN* is controlled by MexT, a transcription activator of the LysR family. The *mexT* gene is located upstream of the *mexEF-oprN* operon (Kohler *et al.*, 1997). In wild-type cells grown under normal conditions, *mexEF-oprN* is not expressed (Kohler *et al.*, 1997). However, overexpression of *mexT* from a plasmid leads to increased levels of MexEF-OprN and increased antibiotic resistance (Kohler *et al.*, 1997; Kohler *et al.*, 1999). Although the majority of LysR-type regulators are not active until they bind an effector molecule (Schell, 1993), a MexT ligand has not been identified. Salicylate, flavone, trigonellin, naringenin, and vanilline, which are effectors of other LysR-type regulators, are unable to induce expression of the pump. Likewise, MexEF-OprN substrates chloramphenicol, norfloxacin, and trimethoprim also fail to induce expression (Kohler *et al.*, 1999). Expression of *mexEF-oprN* is induced by imipenem (Kolayli *et al.*, 2004) and a quorum sensing inhibitor (Hentzer *et al.*, 2003). However, it has not been demonstrated that this induction occurs via MexT. In many wild-type *P. aeruginosa* strains, induction of *mexEF-oprN* may be impossible due to inactivating mutations in *mexT* (Maseda *et al.*, 2000). Evidence is consistent with the existence of an unidentified suppressor, MexS, which prevents MexT from activating *mexEF-oprN* expression. According to this model, expression of *mexEF-oprN* requires mutations both to activate MexT and inactivate its suppressor MexS (Maseda *et al.*, 2000).

A possible candidate for MexS is the oxidoreductase/dehydrogenase homologue Qrh (Sobel *et al.*, 2005). The gene *qrh* (PA2491) is adjacent to *mexT*, but transcribed in the opposite direction. Expression of PA2491 is constitutive but can be further activated by MexT (Kohler *et al.*, 1999). Furthermore, disruption of PA2491 leads to an increase in MexEF-OprN and multidrug resistance (Sobel *et al.*, 2005). It is possible that PA2491 and MexEF-OprN play a common role in detoxification of cellular metabolites, and that such metabolites could be the MexT effector molecule (Sobel *et al.*, 2005).

MexT is also involved in the regulation of other genes including *mexAB-oprM* (Maseda *et al.*, 2004) and *oprD* (encoding a porin that facilitates uptake of basic amino acids and the antibiotic imipenem) (Kohler *et al.*, 1999; Ochs *et al.*, 1999). MexT may also play a role in expression of quorum sensing regulated genes. Overexpression of the MexEF-OprN efflux system results in decreased production of pyocyanin, elastase, and rhamnolipids as well as decreased swarming (Kohler *et al.*, 2001). Additionally, loss of the VsqR quorum sensing and virulence regulator causes a decrease in *mexEF-oprN* expression in conditions of oxidative stress (Juhas *et al.*, 2004).

As more information emerges about regulation of the *P. aeruginosa* efflux systems, the picture becomes increasingly complex. In fact, expression of the pumps may be subject to coordinate regulation (Li *et al.*, 2000). Expression of the *mexCD-oprJ* and *mexEF-oprN* operons is inversely related to *mexAB-oprM* expression. However, increased production of MexCD-OprJ and MexEF-OprN cannot compensate for the loss of MexAB-OprM. At this point, it is unclear if there is global regulation of the efflux pumps or if loss of MexAB-OprM leads to accumulation of compounds that trigger expression of the other multidrug efflux pumps (Li *et al.*, 2000).

Many organisms use two component systems to regulate expression of efflux systems. Two component systems involve a sensor histidine kinase and a response regulator that activates or represses target genes in response to environmental conditions (Stock *et al.*, 2000). The *Staphylococcus aureus* AlsSR system regulates NorA, a MFS pump (Fournier *et al.*, 2000). AdeSR, a two component system in *Acinetobacter baumanii*, regulates RND pump AdeABC (Magnet *et al.*, 2001). Similarly, the *E. coli* RND efflux system MdtABC is regulated by the BaeSR two component system (Baranova and Nikaido, 2002; Nagakubo *et al.*, 2002). The EvgAS system of *E. coli* regulates two efflux pumps: RND pump YhiUV (Nishino and Yamaguchi, 2002) and MFS pump EmrKY (Nishino and Yamaguchi, 2001a). *Stenotrophomonas maltophilia* RND efflux pump SmeABC is regulated by the SmeSR two component system. Except for NorA of *S. aureus* and YhiUV of *E. coli*, the genes encoding all of the above two component systems are located upstream or downstream of the efflux operon. Two component systems may regulate a range of genes in addition to efflux systems. For example, increased SmeSR expression is also associated with increased β-lactamase production in *S. maltophilia* (Li *et al.*, 2002). Also, *E. coli* two component system BaeSR is involved in regulation of the *yicO* and *ygcL* genes (Baranova and Nikaido, 2002). Regulation of bacterial drug efflux pumps is subject to many levels of control by transcriptional regulatory proteins at the global and local levels. Examining how an efflux pump is regulated may provide insight into its natural function.

1.4 Multidrug efflux pumps in the *B. cepacia* complex

Only recently has there been evidence that active efflux is responsible for some of the multidrug resistance observed in members of the *B. cepacia* complex. Burns *et al.* described a strain of *B. cenocepacia*, isolated from a CF patient, that is resistant to chloramphenicol, trimethoprim, ciprofloxacin, β-lactams, and aminoglycosides (Burns *et al.*, 1989a). Later, the DNA fragment conferring resistance to three of these antibiotics, chloramphenicol, trimethoprim, and ciprofloxacin, was subcloned and the nucleotide sequence was analyzed (Burns *et al.*, 1996). Now, the entire efflux gene cluster has been sequenced and found to contain five open reading frames (Nair *et al.*, 2004). The *opcM* gene is homologous to *oprM*, which encodes the outer membrane component of the *P. aeruginosa* MexAB-OprM RND efflux system. Like OprM, OpcM appears to localize to the outer membrane (Burns *et al.*, 1996). Upstream of *opcM* are two efflux genes *ceoB* and *ceoA* (Nair *et al.*, 2004). The *ceoA* and *ceoB* genes show the highest degree of homology to *mexE* and *mexF* respectively, the membrane fusion and inner membrane transporter components of a *P. aeruginosa* RND efflux system. Upstream of the efflux genes is an open reading frame named *llpE* (lipase-like protein for efflux) and a divergent LysR-type transcriptional regulator named *ceoR* (Nair *et al.*, 2004). LlpE is a novel discovery as there are currently no counterparts involved in characterized efflux systems. The sequence of *llpE* suggests it is a member of the α/β hydrolase enzyme family, which includes lipases, esterases, carboxypeptidases and haloperoxidases (Nair *et al.*, 2005). LlpE is clearly not essential for drug efflux, since deletion of *llpE* does not influence antibiotic susceptibility (Nair *et al.*, 2005). RT-PCR results confirmed that *llpE*, *ceoA*, *ceoB*, and *opcM* are cotranscribed (Nair *et al.*, 2004).

Expression of the three structural genes *ceoAB-opcM* in *B. cenocepacia* strain CEP511 confers resistance to chloramphenicol, trimethoprim, and ciprofloxacin, but not ceftazidime or gentamicin. In addition to antibiotics, when expressed in *B. multivorans* 249-2, the CeoAB-OpcM efflux system can also extrude the siderophore salicylate (Nair *et al.*, 2004). In fact, *ceoA* expression in *B. cenocepacia* clinical isolate PC121 is induced by the presence of pump substrates chloramphenicol and salicylate. Expression of a *ceoR-lacZ* promoter fusion also increases in the presence of chloramphenicol and salicylate (Nair *et al.*, 2004). Perhaps the coregulation of *ceoR* and *ceoAB-opcM* prevents levels of the efflux pump from getting too high.

The CeoAB-OpcM efflux system appears to be somewhat widespread in the *B. cepacia* complex. At least some of the components were amplified from genomovars I, II, III, and IV while none of the components were amplified from genomovar V strains (Nair *et al.*, 2004). Homologues of *llpE* are present in genomovars I, III, IV, and VI (Nair *et al.*, 2005).

Wigfield *et al.* discovered a second efflux system in the *B. cepacia* complex. They identified BcrA, an immunodominant antigen of *B. cenocepacia*, which belongs to the 14-transmembrane domain subfamily of MFS efflux pumps (Wigfield *et al.*, 2002). Overexpression of *bcrA* in *E. coli* cells causes a four-fold increase in resistance to tetracycline and an eight-fold increase in resistance to nalidixic acid. Two potential transcriptional regulators of *bcrA* were identified: a downstream gene encoding a LysR-type transcriptional regulator and an upstream gene encoding a member of the multiple antibiotic resistance (*mar*) family (Wigfield *et al.*, 2002).

A MATE family efflux pump was recently found in *B. vietnamiensis* (Fehlner-Gardiner and Valvano, 2002). Following transposon mutagenesis, a polymyxin B-sensitive strain was found to have an insertion in the *norM* gene. Surprisingly, polymyxin B sensitivity occurs only in presence of tetracycline. The mechanism for the tetracycline-dependent increase in sensitivity remains unknown (Fehlner-Gardiner and Valvano, 2002). Although polymyxin B is the only identified substrate in *B. vietnamienis*, *norM* expression is sufficient to restore norfloxacin resistance in an *E. coli* mutant. NorM is conserved in at least genomovars I through VI (Fehlner-Gardiner and Valvano, 2002). Together, the discovery of the NorM, BcrA, and CeoAB-OpcM efflux systems suggests that active efflux has been overlooked as an important resistance mechanism in the *B. cepacia* complex.

1.5 Research Objectives

The *Burkholderia cepacia* complex is best known for its ability to infect the lungs of cystic fibrosis patients. The widespread antibiotic resistance of the *B. cepacia* complex raises great concern regarding the treatment of such infections. The long-held belief that membrane impermeability is the major mechanism of resistance has been challenged by the discovery of multidrug efflux pumps (Burns *et al.*, 1996; Fehlner-Gardiner and Valvano, 2002; Wigfield *et al.*, 2002). We hypothesize that multidrug efflux pumps play a large role in this resistance. The goal of this research project is to assess the contribution of multidrug efflux pumps to the overall resistance of *B. cenocepacia* clinical isolates.

The identification of putative efflux systems in the *B. cepacia* complex was accomplished by searching the genome of *B. cenocepacia* strain J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) using the sequences of efflux genes from closely related organisms. By this method, six open reading frames (ORFs) with homology to RND transporters were found. Several strategies were employed to characterize the contribution of each RND system.

The ability to knock-out genes through insertion of an antibiotic resistance cassette is limited by the *B. cepacia* complex's intrinsic resistance. Also, the presence of a resistance marker could interfere with detection of an antibiotic sensitive phenotype. Therefore, a Flp-FRT recombinase system was developed and used to create a markerless deletion in one of the RND transporters. The minimum inhibitory concentrations (MICs) of various antibiotics were determined to compare the deletion mutant and wild-type cells. However, no change in antimicrobial susceptibility was observed, leading us to believe that this pump may not be expressed under normal laboratory conditions.

RT-PCR experiments revealed that this efflux system was not expressed. Therefore, we attempted to identify pump substrates by overexpressing the efflux system and looking for a corresponding increase in antibiotic resistance. The structural genes, encoding the membrane fusion, RND transporter, and outer membrane proteins, were cloned and introduced into *E. coli* and *B. cenocepacia*. We also cloned the putative transcription activator of this efflux system and introduced it into *B. cenocepacia* hoping to increase expression of the structural genes.

Expression of the six putative RND efflux systems was characterized using RT-PCR. We examined the effect of potential inducers, salicylate and chloramphenicol, on expression levels. We also looked for changes in efflux pump expression at various stages of growth.

Chapter 2: Materials and Methods

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

2.1.1 Burkholderia cepacia and Escherichia coli strains

A variety of *E. coli* and *B. cepacia* complex strains were used in this study. Strains and their relevant characteristics are given in Table 2.1.

2.1.2 Plasmid vectors

Cloning vectors and recombinant plasmids constructed are listed in Table 2.2, along with their relevant properties.

2.1.3 Growth Conditions

Bacteria were grown in Luria-Bertani (LB) or ½ LB medium (Sambrook *et al.*, 1989). *E. coli* cells were grown at 37°C with shaking at 250 rpm. *B. cepacia* complex cells were grown at 30°C with shaking at 250 rpm. When necessary for selection, antibiotics were added to the growth medium (liquid and solid) in the following concentrations: for *E. coli*: 100 µg/mL ampicillin, 30 µg/mL chloramphenicol, 50 µg/mL kanamycin, and 100 µg/mL trimethoprim; for *B. cenocepacia* J2315: 300 µg/mL chloramphenicol and 500 µg/mL trimethoprim; for *B. cenocepacia* CEP511, K56-2, and 715j and for *B. vietnamiensis* DB01: 80 µg/mL chloramphenicol and 100 µg/mL trimethoprim.

2.1.4 Preparation of frozen bacterial stocks

Table 2.1 Bacterial strains used in this study

Strain	Description	Reference or Source
E. coli		
DH5α	F$^-$ Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r$_k^-$, m$_k^+$) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
One Shot TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
B. cepacia complex		
715j	*B. cenocepacia* CF isolate	(McKevitt and Woods, 1984)
CEP511	*B. cenocepacia* CF isolate	(Mahenthiralingam *et al.*, 1996)
DB01	*B. vietnamiensis* phthalate degrading isolate	(Bull and Ballou, 1981)
J2315	*B. cenocepacia* CF isolate	(Govan *et al.*, 1993)
K56-2	*B. cenocepacia* CF isolate	(Darling *et al.*, 1998)
Δefflux 5	Derivative of CEP511 with a 978 bp (*Sal*I – *Sal*I) deletion within the efflux 5 transporter	This study

Table 2.2 Plasmids and cloning vectors used in this study

Plasmid	Relevant Characteristics*	Reference
pBAD33	Low copy number, arabinose-inducible promoter, chloramphenicol resistance marker, does not replicate in *B. cepacia* complex strains	(Guzman *et al.*, 1995)
pBAD33 + efflux 1 FRT	Efflux 1.4 L, R PCR product cloned into pBAD33 (*Hind*III - *Sac*I) and with insertion of the FRT cassette from pPS854-Tp (*Sal*I)	This study
pBAD33 + efflux 3 FRT	Efflux 3.1 L, R PCR product cloned into pBAD33 (*Hind*III - *Sac*I) and with insertion of the FRT cassette from pPS854-Tp (*Sal*I)	This study
pBAD33 + efflux 5 FRT	Efflux 5.2 2L, 2R PCR product cloned into pBAD33 (*Xba*I - *Sph*I) and with insertion of the FRT cassette from pPS854-Tp (*Sal*I)	This study
pBBR1MCS	Broad host-range, mobilizable, medium copy number, blue/white screening, chloramphenicol resistance marker	(Kovach *et al.*, 1994)
pBBR1MCS + efflux 5	Efflux 5 stuctural genes and ~1000 bp upstream cloned into pBBR1MCS (*Bam*HI – *Bam*HI)	This study
pBBR1MCS + efflux 5 reg	Efflux 5 putative regulator cloned into pBBR1MCS (*Pst*I - *Xba*I)	This study
pBBR1MCS-Tp	Derivative of pBBR1MCS with a trimethoprim resistance cassette	(DeShazer and Woods, 1996)
pBBR1MCS-Tp + efflux 5	Efflux 5 stuctural genes and ~1000 bp upstream cloned into pBBR1MCS-Tp (*Bam*HI - *Bam*HI)	This study
pBBR1MCS-Tp + efflux 5 reg	Efflux 5 putative regulator cloned into pBBR1MCS-Tp (*Pst*I - *Xba*I)	This study
pCR 2.1 TOPO	TOPO TA cloning vector for direct insertion of PCR products, blue/white screening, ampicillin and kanamycin resistance markers	Invitrogen
pFLP2	Flippase gene under control of λ promoter, Ts cI repressor, *sacB*, ampicillin resistance marker	(Hoang *et al.*, 1998)
pFLP2Cm	*Kpn*I - *Pst*I fragment of pFLP2 (including *Flp*, *cI*, *sacB*) cloned into pBBR1MCS	This study
pPS854	Ampicillin resistance marker, FRT cassette	(Hoang *et al.*, 1998)
pPS854Tp	Derivative of pPS854 with trimethoprim resistance marker from pBBR1MCS-Tp inserted into *Eco*RI site within FRT cassette	This study

* Primers used in the construction of the plasmids are described in Table 2.3.

Bacteria were grown overnight on LB agar with appropriate antibiotics, then suspended in 1 mL of LB + 20% glycerol (vol/vol). Once prepared, strains were stored at -80°C.

2.2 Transformation methods

2.2.1 Transformation of E. coli

E. coli DH5α subcloning efficiency and One Shot TOP10 chemically competent cells were commercially obtained (Invitrogen, Carlsbad, CA) and heat-shocked according to product specifications.

2.2.2 Transformation of B. cepacia complex

B. cepacia complex strains were grown overnight on LB agar. Then, cells were suspended in 500 µL of 10% glycerol (vol/vol), transferred into a sterile 1.5 mL microfuge tube, and centrifuged at 4800 rpm. This wash was repeated two additional times with 250 µL and 100 µL of 10% glycerol. Following the washes, cells were resuspended in 80 µL of 10% glycerol, mixed with an appropriate amount of plasmid DNA, and electroporated using BIO-RAD (Hercules, CA) MicroPulser setting Ec1 (0.1 cm cuvette, 1.8 kV). After electroporation, cells were resuspended immediately in 500 µL SOC medium (Sambrook *et al.*, 1989) and incubated for one hour at 30°C with shaking at 250 rpm. Following incubation, cells were plated in 100 µL volumes on LB agar with selective antibiotics.

2.3 DNA analysis

2.3.1 Isolation of plasmid DNA

For isolation of plasmid DNA from *E. coli* and the *B. cepacia* complex, the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) was used as specified by the manufacturer's recommendations.

2.3.2 Isolation of chromosomal DNA

Genomic DNA was isolated from the *B. cepacia* complex using a previously described phenol/chloroform extraction (Ausubel *et al.*, 1999).

2.3.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify DNA fragments for cloning and sequencing reactions. PCR was also used to confirm the insertion or excision of antibiotic resistance cassettes during mutagenesis. Oligonucleotide primers used in this study were purchased from Sigma (St. Louis, MO) and are listed in Table 2.3. Reactions were performed in 20 μL volumes in 0.2 mL tubes using the Eppendorf Mastercycler gradient thermocycler (Westbury, NY). A standard PCR reaction contained 1X PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward and reverse primers, and *Taq* polymerase (gift from M.A. Pickard, University of Alberta). When necessary, 0-10% dimethyl sulfoxide (DMSO) was added to the reaction mixture. For template DNA, 5 μL of a 1/50 chromosomal DNA dilution or 1 μL of a 1/50 dilution of plasmid DNA was used. A typical PCR program consisted of a 2 minute denaturation at 94°C followed by 30 cycles of a 45 second denaturation step at 94°C, a 30

Table 2.3 Oligonucleotide primers used in this study

Primer	Sequence (5'-3')*	PCR Conditions	Use
Efflux 1 1596R**	GTGGTTCACGCCGCTCGT	60°C, 2% DMSO	RT-PCR within efflux 1 transporter
Efflux 1 443F	GATGTCGAAGGAGGACCTGA	60°C, 2% DMSO	RT-PCR within efflux 1 transporter
Efflux 1.4 L	AAAAGCTTGCAGGTCACGAAGTCG	60°C, 2% DMSO	Construction of efflux 1 transporter deletion
Efflux 1.4 R	AAGAGCTCCGTCTCGCCGCTGA	60°C, 2% DMSO	Construction of efflux 1 transporter deletion
Efflux 2 1775F	ACATTTCCGACTACCTGTTG	54°C, 8% DMSO	RT-PCR within efflux 2 transporter
Efflux 2 2874R**	CCCCATCTTCTCCGTCG	54°C, 8% DMSO	RT-PCR within efflux 2 transporter
Efflux 3 1071R**	AACAGCCACATCACGCAA	60°C, 6% DMSO	RT-PCR within efflux 3 transporter
Efflux 3 471F	CGATTACCTGAACCGCTAC	60°C, 6% DMSO	RT-PCR within efflux 3 transporter
Efflux 3.1 L	ATAAGCTTGTGCTGACCGCCGTATT	60°C, 2% DMSO	Construction of efflux 3 transporter deletion
Efflux 3.1 R	TTGAGCTCATAACACCCCTCTGTC	60°C, 2% DMSO	Construction of efflux 3 transporter deletion
Efflux 4 1682F	AATTCATCCCGAGCCTCAA	60°C, 8% DMSO	RT-PCR within efflux 4 transporter
Efflux 4 2737R	GCCCGTCCTTCACGTTGTT	60°C, 8% DMSO	RT-PCR within efflux 4 transporter
Efflux 5 1627F	CAGTTCCGACCGCTACCA	60°C, 2% DMSO	RT-PCR within efflux 5 transporter
Efflux 5 2211R	GGTACGAGCTGACGGGATAG	60°C, 2% DMSO	RT-PCR within efflux 5 transporter
Efflux 5.2 2L	AATCTAGACCGCTGGAAGAGGCGA	60°C, 5% DMSO	Construction of efflux 5 transporter deletion
Efflux 5.2 2R**	AAGCATGCGAGCGTCACCGACATC	60°C, 5% DMSO	Construction of efflux 5 transporter deletion, RT-PCR within efflux 5 transporter
Efflux 5.2 3L	TAGGATCCTGAAAAGCCTGTTCC	60°C, Buffer 3, 6% DMSO	Overexpression of efflux 5 structural genes
Efflux 5.2 4R	TTGGATCCGTCTACTACTACATCA	60°C, Buffer 3, 6% DMSO	Overexpression of efflux 5 structural genes
Efflux 5reg 2L	TACTGCAGCATGGAAGGTATCGCAT	60°C, Failsafe PreMix H	Overexpression of efflux 5 regulator
Efflux 5reg 2R	ATTCTAGAGTTCACGTCGTTGCCGA	60°C, Failsafe PreMix H	Overexpression of efflux 5 regulator
Efflux 6 1143F	CACGTTCTCGCTGCTGCT	60°C, Failsafe PreMix H	RT-PCR within efflux 6 transporter
Efflux 6 2002R**	CTTCATCGCGCCGTACTT	60°C, Failsafe PreMix H	RT-PCR within efflux 6 transporter
FRT 1.L	GAGCTCGAATTGGGGATCTTG	60°C	PCR verification of FRT insertion and excision
FRT 1.R	GAGCTCGAATTAGCTTCAAAAG	60°C	PCR verification of FRT insertion and excision
RecA 130F	ATCCAGGTCGTCTCCACG	60°C, 2% DMSO	RT-PCR positive control within *recA*
RecA 921R**	GATCTTCTCGCCGTTGTAG	60°C, 2% DMSO	RT-PCR positive control within *recA*

L and F refer to the 5' primer

R refers to the 3' primer

* underlined sequences indicate engineered resistriction endonuclease sites

** indicates the oligonucleotide primer used for first strand cDNA synthesis second annealing step at 50-65°C, and a 1 minute extension period at 72°C. Finally, 2 minutes at 68°C provided additional extension time.

In some cases, the Failsafe PCR PreMix Selection Kit (Epicentre Biotechnologies, Madison, WI) was used. Each reaction contained 1.5 µL of a 1/50 dilution of chromosomal DNA, and 0.25 µL (0.625 units) of a PCR enzyme mix containing a proof reading enzyme or 1 µL of *Taq* polymerase. After an initial denaturation at 94°C for 2 minutes, 0.5 µL of each 50 µM primer was added. Then, a standard PCR reaction was used as described above.

For amplification of long regions of DNA (>3kb), the Expand Long Template PCR System was used (Roche Diagnostics, Indianapolis, IN). This system uses a mix of *Taq* DNA polymerase and a proofreading polymerase. Reactions were prepared using the supplied PCR buffer 3 according to the manufacturer's specifications. Thermal cycling was performed as directed in the product guide. An initial elongation time of 8 minutes was used and this extension time was increased gradually over the course of the reaction to ensure maximum amplification of target DNA.

2.3.4 Gel electrophoresis of DNA

DNA samples were suspended in loading buffer containing 0.25% bromophenol blue and 40% sucrose (Sambrook *et al.*, 1989). Samples were subjected to electrophoresis in 0.8% agarose, 1X TAE gels. Electrophoresis was carried out at 100 – 130 V. The 1 kb Plus DNA ladder from Invitrogen (Carlsbad, CA) was used as a molecular weight marker. Following electrophoresis, gels were stained in ethidium bromide for visualization with ultraviolet light.

2.3.5 Purification of DNA from agarose gels

For isolation and purification of DNA from agarose gels, the Geneclean II Kit was used (Q-BIOgene, Irvine, CA). Briefly, an agarose gel fragment was melted in NaI at 55°C. Then a silica matrix was added to bind DNA out of the solution. The silica-DNA complex was pelleted by centrifugation and washed with a solution of NaCl, Tris, EDTA, and ethanol. Again, the silica-DNA complex was pelleted and dried to remove the ethanol. At this point, the silica-DNA complex was suspended in sterile water to elute the DNA. After centrifugation, the supernatant, containing the DNA, was removed and transferred into a clean tube for immediate use or storage at 4°C.

2.3.6 Cloning of DNA

General molecular techniques were performed as detailed by Sambrook *et al.* (1989). Restriction endonuclease digestion was performed using enzymes from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. For ligation reactions, an insert to vector ratio of approximately 3:1 was used. Reactions were carried out in 10-15 μL volumes using T4 DNA ligase (Promega, Madison, WI) at room temperature (directional cloning) or 16°C (non-directional cloning) for 4-16 hours. In some cases, PCR products amplified with *Taq* polymerase were first cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and subsequently cloned into suitable vectors for use in the *B. cepacia* complex.

2.3.7 DNA sequence analysis

Automated DNA sequencing was carried out using the DYEnamic ET kit (Amersham Biosciences, Piscataway, NJ). The sequencing reaction contained 1-3 µL of template DNA (plasmid or purified PCR product), 4 µL of ET reagent, 1 µL of 5 µM primer, and sequencing buffer (20 mM Tris, pH 9.0, 5mM $MgCl_2$) to a final volume of 20 µL. The standard reaction program consisted of 35 cycles of a 20 second denaturation step at 95°C, a 15 second primer annealing step at 50°C, and a 60 second extension step at 60°C. Reactions were stopped by the addition of 2 µL sodium acetate/EDTA. DNA was precipitated at -20°C for 15 minutes after the addition of 80 µL 95% ethanol. After centrifugation, the resulting pellets were washed with 500 µL 70% ethanol, stored at -80°C, and provided to the Molecular Biology Service Unit (MBSU, University of Alberta) for electrophoresis on an ABI PRISM 377 or 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

2.4 RNA analysis

2.4.1 RNA isolation

One mL of an overnight culture of *B. cenocepacia* was used to inoculate 25 mL of LB medium. The cultures were grown at 30°C with shaking at 250 rpm to an OD_{600} of 0.5-0.7. At this point, 3 mL of culture were removed for RNA isolation. The MasterPure RNA Purification Kit (Epicentre, Madison, WI) was used according to manufacturer's instructions with one modification. Following the first precipitation of nucleic acids, the pellet was resuspended and digested with 5 units of DNaseI for 30 minutes at 37°C. Then, an additional 5 units of DNaseI were added for a second 30 minute digestion at 37°C. Upon completion of the RNA isolation procedure, RNA was suspended in 30 µL of sterile DEPC-treated water and kept at -20°C overnight to allow the RNA to go into solution. RNA was then quantified by spectrophotometry, measuring absorbance at 260 nm (1 absorbance unit = 40 μg/mL RNA) (Sambrook *et al.*, 1989). The quality of RNA was determined by the ratio of A_{260}/A_{280}. Pure RNA has an A_{260}/A_{280} ratio of 2.0. A ratio of < 2.0 indicates that the RNA sample is contaminated with protein or phenol (Sambrook *et al.*, 1989). For long-term storage, RNA was stored in sterile DEPC-treated water and frozen at -80°C. Isolation of RNA from cells in late-logarithmic or stationary phase occurred as described above with the following exceptions: cells were grown in ½ LB medium at 30°C with shaking at 250 rpm, and 1 mL aliquots were removed when the culture reached an OD_{600} of 1.2 (late-logarithmic phase) and again after growth overnight (stationary phase).

2.4.2 Reverse-transcriptase PCR (RT-PCR)

The primers for first strand cDNA sythesis and PCR were designed to bind specifically to each of the six putative RND transporter genes. Specificity was confirmed by sequencing the PCR products as described above. As a positive control, the *recA* gene was amplified. The oligonucleotide primers used for cDNA synthesis and PCR are described in Table 2.3.

Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to generate cDNA. Reaction mixtures contained 1 μg of total RNA, 2 μM of gene specific primer, 1 μL of DMSO, 1 μL of 10 mM dNTPs, and DEPC-treated water to a final volume of 10 μL. This reaction was heated at 65°C for 5 minutes in an Eppendorf Mastercycler gradient thermocycler, then cooled to 4°C for at least 1 minute.

Next, appropriate amounts of 10X RT buffer, 25 mM $MgCl_2$, 0.1M DTT, RNase Out, and Superscript III reverse transcriptase were added according to the manufacturer's instructions. A negative control was prepared as described above, except without the addition of Superscript III. Synthesis occurred for 1 hour at 55°C (or 2 hours for efflux 5 cDNA synthesis) and was terminated by heating to 85°C for 5 minutes. The cDNA was used immediately for PCR or stored at 4°C.

The yield of RNA isolated from late log or stationary phase cells was much lower than that of the mid-log phase cell. As a result, first strand cDNA synthesis for these growth conditions was performed using only 0.75μg total RNA. The cDNA synthesis reaction for all transporter genes was extended to 2 hours at 55°C.

Following synthesis, 2 μL of cDNA was used as the template for a PCR reaction. PCR was performed as described above except that primers were added after the initial 2 minute, 94°C denaturation step and that the amplification continued for 28 cycles instead of 30.

2.5 Minimum inhibitory concentration (MIC) assay

Antibiotics were purchased from various commercial sources, including Sigma (St. Louis, MO) and Fisher (Fairlawn, NJ). Stock solutions of antibiotics were prepared according to the solubilities listed in the MERCK index (Budavari *et al.*, 1996) to concentrations of 4 mg/mL and 8 mg/mL. Antibiotics were serially diluted into 96 well plates containing 100 μL of LB per well. After dilution, each well contained 100 μL of antibiotic solution, with half the concentration of the previous well.

A single colony of *B. cenocepacia* was used to inoculate 5 mL of LB and grown overnight at 30°C. One mL of the overnight culture was used to inoculate 25 mL of LB and incubated at 30°C until reaching a final OD_{600} of approximatey 1.0. Then, 5 µL of culture was used to inoculate each well containing antibiotic solution. Plates were incubated on a shaker at 30°C for approximately 24 hours. The minimum inhibitory concentration (MIC), or the lowest antibiotic concentration that completely inhibited bacterial growth, was determined by measuring OD_{600} with a Wallac Victor^2 multilabel counter (Perkin Elmer, Shelton, CT).

2.6 Computer-assisted sequence analysis

For raw sequence handling, the GeneJockey Sequence Processor (BIOSOFT, Ferguson, MO) was used. Oligonucleotide primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) and Primers (Anderson and Bristol, UCLA, Los Angeles, CA). For automated sequence viewing and assembly, both ABI PRISM EditView (Perkin Elmer) and GeneTool 2.0 (BioTools, Edmonton, AB) were used. Putative RND transporters were identified by performing BLAST (Altschul *et al.*, 1990) searches of the *B. cenocepacia* database (http://www.sanger.ac.uk/Projects/B_cenocepacia/) with the sequences of known efflux systems in related organisms. The homology of the putative *B. cenocepacia* RND efflux systems to other known RND efflux pumps was determined using BLASTP searches of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Chapter 3: Results

3. RESULTS

3.1 Identification of putative RND transporters in *B. cenocepacia*

We wanted to assess the contribution of multidrug efflux pumps to the overall antibiotic resistance of *B. cenocepacia*. RND family transporters are considered the most relevant multidrug efflux pumps in terms of resistance to clinically important agents (Poole, 2004). Therefore a logical starting point for the study of *B. cenocepacia* multidrug resistance was to search for members of the RND family. To identify putative RND efflux systems, the genome of *B. cenocepacia* J2315 was searched using sequences from known efflux pumps in related organisms such as *E. coli*, *P. aeruginosa*, and *N. gonorrhoeae*. Six ORFs were found with homology to RND transporters. Since these putative efflux systems have not been given official names, they will be referred to as efflux system numbers 1 through 6. With the exception of efflux system 3, each of the putative RND transporters was located along with ORFs encoding putative membrane fusion proteins, outer membrane proteins, and transcription regulators (Figure 3.1). To identify which known efflux pumps the putative *B. cenocepacia* ORFs were most similar to, BLASTP searches were performed.

Efflux system 1 contained each member of a tripartite efflux system similar to the *P. aeruginosa* MexAB-OprM multidrug efflux pump (Poole *et al.*, 1993b). The RND transporter was 77% similar and 63% identical at the protein level to MexB (accession number AAG03815). Immediately downstream of the transporter gene was an ORF encoding an outer membrane protein that was 69% similar and 54% identical to *P. aeruginosa* OprM (accession number AAG03816). Immediately upstream of the transporter, a gene encoding a membrane fusion protein that was 69% similar and 55%

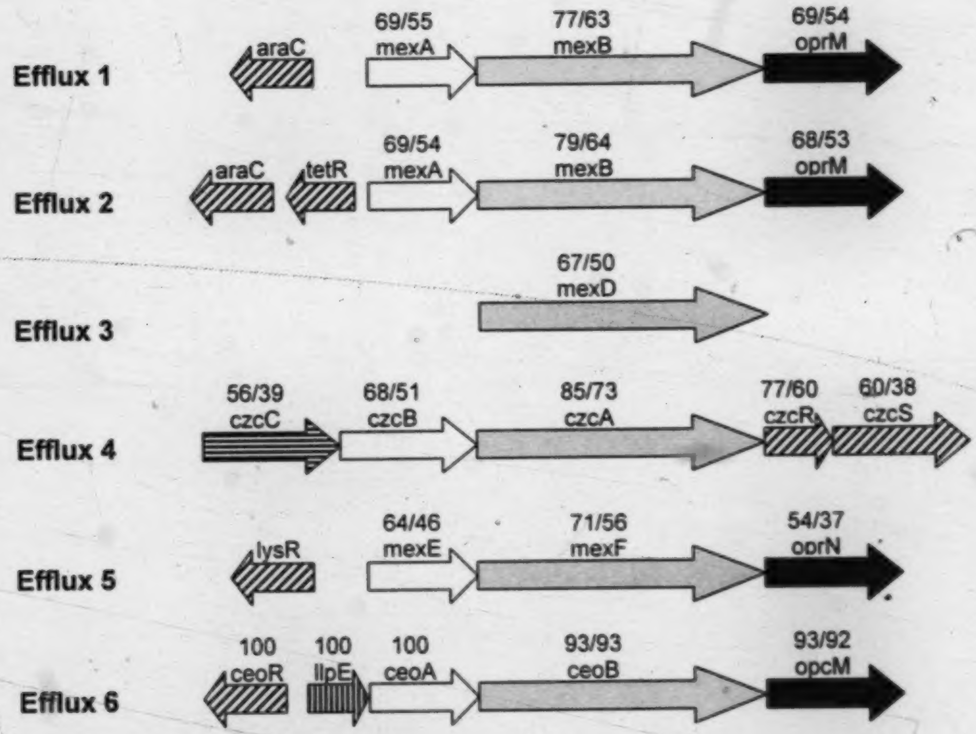
Efflux 1
araC
69/55
mexA
77/63
mexB
69/54
oprM
Efflux 2
araC
tetR
69/54
mexA
79/64
mexB
68/53
oprM
Efflux 3
67/50
mexD
Efflux 4
56/39
czcC
68/51
czcB
85/73
czcA
77/60
czcR
60/38
czcS
Efflux 5
lysR
64/46
mexE
71/56
mexF
54/37
oprN
Efflux 6
100
ceoR
100
llpE
100
ceoA
93/93
ceoB
93/92
opcM

Figure 3.1 Six putative RND efflux systems in *B. cenocepacia* J2315. Potential efflux pumps were identified by searching the genome sequence of *B. cepacia* J2315 with DNA sequences from known efflux pump genes. ORFs encoding RND transporters (light gray), membrane fusion proteins (white), outer membrane proteins (black), transcription regulators (diagonal lines) were found. For efflux system 4, a putative outer membrane-associated protein was encoded (horizontal lines). For efflux system 6, an *llpE*-homologue was also identified (vertical lines). The closest homologues of each predicted protein product, determined by BLASTP searches, are shown above each ORF (% similarity/identity).

identical to *P. aeruginosa* MexA was identified (accession number AAG03814). A putative regulator, with homology to members of the AraC family (Egan, 2002), is transcribed upstream and in the opposite orientation of the membrane fusion component. The AraC-type regulators are usually activators, but the family also includes some repressors of transcription (Gallegos *et al.*, 1993).

Like efflux system 1, efflux system 2 showed homology to the MexAB-OprM RND pump of *P. aeruginosa* (Poole *et al.*, 1993b). The transporter component of efflux system 2 was homologous to MexB (accession number AAG03815), showing 79% similarity and 64% identity. The membrane fusion component was 69% similar and 54% identical to MexA (accession number AAG03814). An outer membrane protein with 68% similarity and 53% identity to OprM (accession number AAG03816) was encoded downstream of the transporter gene. Unlike efflux system 1, efflux system 2 had two putative regulatory genes nearby, one belonging to the TetR family of repressors (Grkovic *et al.*, 2002) and the other belonging to the AraC family (Egan, 2002).

Efflux system 3 was different than the other five putative RND systems because the transporter gene was not found near ORFs encoding putative membrane fusion and outer membrane proteins. The putative RND transporter was 67% similar and 50% identical to MexD (accession number AAG07986), another multidrug transporter from *P. aeruginosa* (Poole *et al.*, 1996a). Upstream of efflux system 3 was a gene encoding a putative ABC transporter. A putative TonB-dependent outer membrane receptor was located downstream of efflux system 3 (data not shown).

Efflux system 4 did not show homology to a multidrug transporter, but rather the *Ralstonia metallidurans* (*Alcaligenes eutrophus*) heavy metal cation RND transporter

CzcA (85% similarity, 73% identity, accession number CAA67084). A membrane fusion component showing 68% similarity and 51% identity to CzcB (accession number CAA67083) was encoded directly upstream of the CzcA-homolog. Upstream of the membrane fusion component, a protein was encoded with 56% similarity and 39% identity to CzcC (accession number CAA67082). CzcC is an outer membrane-associated protein that may facilitate transport across the outer membrane by recruiting a hypothetical outer membrane protein (Rensing *et al.*, 1997). Encoded downstream of the RND transporter was a sensor histidine kinase (HK) and response regulator (RR) pair homologous to the *R. metallidurans* two component system CzcS (60% similarity, 38% identity, accession number CAA67087) and CzcR (77% similarity, 60% identity, accession number CAA67086). The Czc efflux system is involved in resistance to cobalt, zinc, and cadmium in *R. metallidurans* (Nies *et al.*, 1989; Nies, 1992; van der Lelie *et al.*, 1997).

The efflux system 5 transporter was most homologous to *P. aeruginosa* multidrug transporter MexF (71% similarity, 56% identity, accession number AAG05882) (Kohler *et al.*, 1997). It was located between a membrane fusion component with homology to MexE (64% similar, 46% identical, accession number AAG05881) and an outer membrane component with homology to OprN (54% similar, 37% identical, accession number AAG05883). Upstream of this putative efflux operon was a divergently transcribed regulator. The predicted product of this regulatory gene was homologous to members of the LysR family of transcription activators (Schell, 1993).

The RND transporter from efflux system 6 was also very similar to MexF (80% similar and 64% identical, accession number AAG05882) of *P. aeruginosa* (Kohler *et al.*, 1997), but was most homologous to the CeoB RND pump (93% similarity and identity, accession number AAB58161) of *B. cenocepacia* K61-3 (Nair *et al.*, 2004). The outer membrane component of efflux system 6 was also homologous to the *B. cenocepacia* K61-3 system (Burns *et al.*, 1996), with 93% similarity and 92% identity to OpcM (accession number AAC43969). Continuing this trend, the putative LysR-type regulator encoded upstream of efflux system 6 was 100% identical at the protein level to CeoR (accession number AAG21824) and the putative membrane fusion protein belonging to efflux system 6 was 100% identical to *B. cenocepacia* K61-3 CeoA (accession number AAB58160) (Nair *et al.*, 2004). Efflux system 6 also contains an ORF with a product that is 100% identical to LlpE (accession number AAG21825), a gene that is cotranscribed with the *ceoAB-opcM* efflux pump and encodes a lipase-like protein of unknown function (Nair *et al.*, 2004).

3.2 Construction of a Flp/FRT system for use in the *B. cepacia* complex

Once the six putative efflux pumps were identified, we wanted to determine the contribution of each pump to the overall antibiotic resistance of *B. cenocepacia* J2315. This contribution was to be assessed by creating deletions in the transporter genes, alone and in combination. Then, the minimum inhibitory concentrations (MICs) of various antibiotics against the wild-type and deletion strains could be compared. However, members of the *B. cepacia* complex are known to be extremely antibiotic resistant, which limits the availability of markers for selection of mutants. We were also concerned that the presence of antibiotic resistance markers in the genome could mask an antibiotic sensitive phenotype in the deletion mutants. Therefore, a Flp/FRT system was developed for the creation of markerless mutations in *B. cenocepacia*. Flp/FRT systems make use of the Flp enzyme, a site specific recombinase that promotes recombination at specific 13 bp sites, termed Flp recombinase target (FRT) sites (Cox, 1983). Therefore, a selectable marker carried between two FRT sites can be used to mark a deletion and later be excised by the introduction of the Flp enzyme. These systems require a gene delivery vector, an FRT cassette, and a Flp recombinase producing plasmid.

To deliver the interrupted mutant allele into *B. cenocepacia*, pBAD33 was used (Guzman *et al.*, 1995). This vector was chosen because it does not replicate in members of the *B. cepacia* complex, facilitating selection of homologous recombination events. Also, pBAD33 has a multiple cloning site and a chloramphenicol resistance marker that are useful for cloning and maintaining a gene of interest in *E. coli*.

The previously described plasmid pPS854 (Hoang *et al.*, 1998) was used as the source of the FRT cassette. This plasmid contains the FRT sites flanked with eight restriction endonuclease sites in inverted order, allowing the FRT cassette to be easily inserted into a gene of interest. The FRT cassette contains two FRT sites separated by 68 bp. Between the two FRT sites are *Eco*RI and *Eco*RV restriction endonuclease sites. For use in *B. cenocepacia*, we cloned the trimethoprim resistance marker from pBBR1MCS-Tp into the *Eco*RI site of the pPS854 FRT cassette. The resulting plasmid, pPS854Tp (Figure 3.2 A), was used as the source of the FRT cassette in this study since trimethoprim resistance is one of the few markers that can be easily selected in the *B. cepacia* complex.

High intrinsic β-lactam resistance in the *B. cepacia* complex prevents the use of the Flp recombinase-producing plasmid pFLP2 (Hoang *et al.*, 1998). To address this

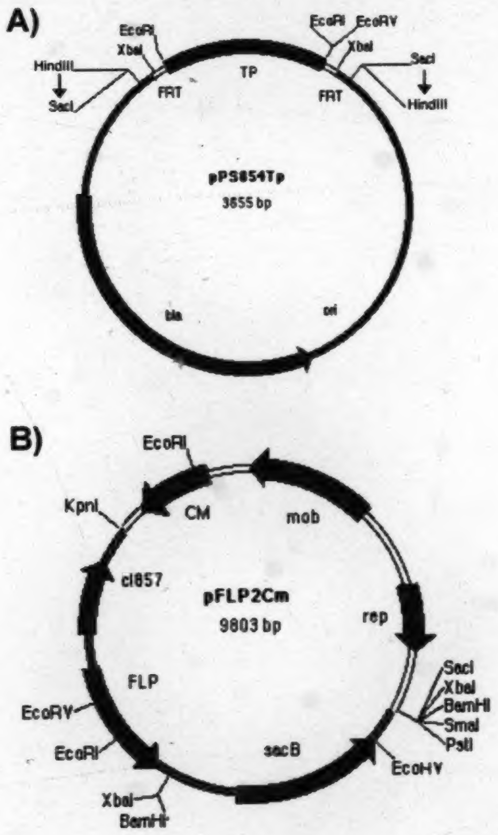
A)
EcoRI
XbaI
HindIII
SacI
TP
FRT
EcoRI
EcoRV
XbaI
SacI
HindIII
FRT
pPS854Tp
3655 bp
bla
ori
B)
EcoRI
KpnI
CM
mob
cI857
pFLP2Cm
9803 bp
rep
SacI
XbaI
BamHI
SmaI
PstI
FLP
EcoRV
EcoRI
sacB
EcoRV
XbaI
BamHI

Figure 3.2 Physical maps of plasmids used in the *B. cepacia* complex Flp/FRT system. (A) The FRT cassette vector pPS854Tp was constructed as described in Section 3.2. The sequence of the FRT cassette has been previously described (Hoang *et al.*, 1998). Only selected restriction enzyme cleavage sites are shown. *Hind*III → *Sac*I, *Hind*III-*Sph*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI-*Sma*I-*Kpn*I-*Sac*I. (B) The pFLP2Cm plasmid was derived as described in Section 3.2. DNA shown in white originates from pBBR1MCS and DNA shown in gray is from pFLP2. Expression of Flp is driven by the strong, rightward λ promoter and is controlled by the cI_{857}-encoded, temperature sensitive λ repressor.

problem, pFLP2Cm was constructed (Figure 3.2 B). A *Kpn*I – *Pst*I fragment from pFLP2 was ligated to pBBR1MCS. This new Flp recombinase-producing plasmid contains the Flp gene expressed from the strong λ promoter. Expression of Flp is regulated by the temperature sensitive cI_{857} λ repressor. The pFLP2Cm plasmid has the broad host range origin of replication from pBBR1MCS, allowing its use in members of the *B. cepacia* complex. It also contains the chloramphenicol resistance marker from pBBR1MCS, which is useful for selection in some *B. cepacia* complex strains. Finally, pFLP2Cm has the *sacB* gene from pFLP2, encoding a levansucrase enzyme that releases glucose from sucrose, creating a byproduct that is toxic for Gram-negative bacteria. Therefore, loss of the plasmid can be selected for following excision of the FRT cassette.

3.3 Isolation of an unmarked *B. cenocepacia* efflux transporter deletion

The application of this Flp/FRT system in *B. cenocepacia* was tested by creating a markerless deletion in the efflux system 5 RND transporter. The general procedure is outlined in Figure 3.3. Approximately 3 kb of the efflux 5 transporter was amplified from *B. cenocepacia* J2315 and cloned into the pBAD33 vector by means of *Xba*I and *Sph*I restriction sites engineered into oligonucleotide primers Efflux 5.2 2R and 2L (Table 2.3). *Sal*I digestion of this plasmid resulted in a 978 bp deletion within the transporter gene. Simultaneously, the FRT cassette from pPS854Tp was inserted into the *Sal*I site to mark the deletion. The resulting construct contained the FRT cassette flanked on each side by 1 kb of *B. cenocepacia* DNA. Similar constructs were made for the efflux 1 (963 bp deletion flanked by 837 bp and 931 bp of DNA) and efflux 3 (968 bp deletion flanked by 584 bp and 1115 bp of DNA) transporter genes as described in Table 2.2.

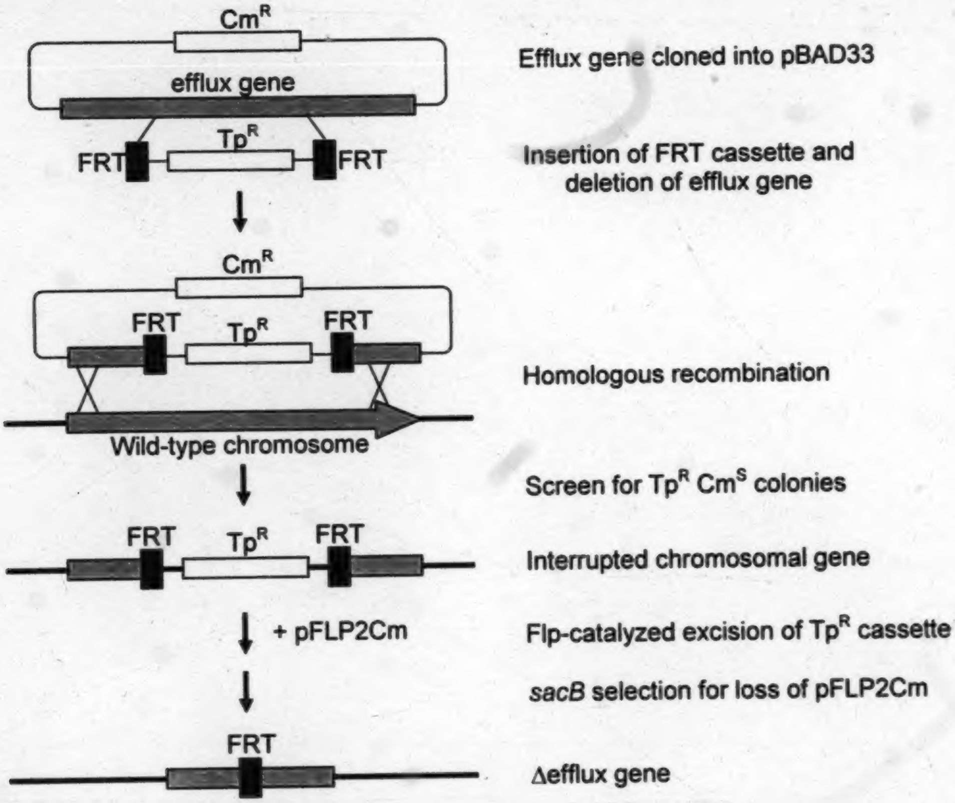
CmR
efflux gene
FRT
TpR
FRT
Efflux gene cloned into pBAD33
Insertion of FRT cassette and deletion of efflux gene
CmR
FRT
TpR
FRT
Homologous recombination
Wild-type chromosome
Screen for TpR CmS colonies
FRT
TpR
FRT
Interrupted chromosomal gene
+ pFLP2Cm
Flp-catalyzed excision of TpR cassette
sacB selection for loss of pFLP2Cm
FRT
Δefflux gene

Figure 3.3 Strategy for isolation of markerless deletions. This system utilizes a trimethoprim resistance cassette flanked by two Flp recombinase target (FRT) sites that can be inserted into any target gene. The interrupted gene, carried by pBAD33, is delivered into the *B. cepacia* complex where it may undergo homologous recombination with the host chromosome. Since pBAD33 does not replicate in the *B. cepacia* complex, any trimethoprim resistant, chloramphenicol sensitive colonies indicate a double recombination event. Once the wild-type copy of the gene has been replaced with the interrupted copy, the flippase gene is provided *in trans* on the broad host range plasmid pFLP2Cm in order to excise the trimethoprim resistance cassette. Loss of pFLP2Cm can be selected for by growth on LB medium containing 5% sucrose.

The next step was to introduce the marked deletion into *B. cenocepacia* via homologous recombination. Since pBAD33 does not replicate in members of the *B. cepacia* complex, any resistant colonies must be recombinants. Colonies that are resistant to both trimethoprim and chloramphenicol represent a single recombination event, resulting in the integration of pBAD33 and the marked deletion into the host chromosome. Colonies that are trimethoprim resistant but chloramphenicol sensitive indicate that a double recombination event has occurred in which the wild-type transporter gene has been replaced by the marked deletion.

Originally, we planned to introduce efflux deletions into *B. cenocepacia* J2315. This organism is a clinical isolate that is well known for having extremely high levels of antibiotic resistance. Also, the genome of this organism has been sequenced and was used to identify and clone the putative efflux genes. However, we have never been able to observe integration of DNA into the J2315 chromosome by homologous recombination. This may be due to difficulties in introducing pBAD33 into J2315. The transformation efficiency of *B. cenocepacia* J2315 is negligible compared to other strains (Mahenthiralingam *et al.*, 2000b). The decreased amount of pBAD33 getting into the cells may have limited the chance of recombination events. An additional complication is strain J2315's high level of background resistance to trimethoprim, which made it difficult to select recombinants. As such, we experimented with the use of other members of the *B. cepacia* complex that may be more amenable to the Flp/FRT system. Our lab has had previous success creating mutants in *B. vietnamiensis* DB01. Some progress was made in creating a deletion of the efflux 3 transporter in this strain. A deleted copy of the gene was integrated into the host genome. However, we were unable to introduce the Flp-carrying plasmid pFLP2Cm due to the inducible chloramphenicol resistance of DB01. Similarly, inducible chloramphenicol resistance also interfered with the use of pFLP2Cm after integration of the efflux 5 deletion in *B. cenocepacia* K56-2. *B. cenocepacia* 715j was compatible with the antibiotic resistance markers used in the Flp/FRT system. An efflux 3 transporter deletion was integrated into the chromosome of this strain. However, work in strain 715j was not continued because success was achieved using *B. cenocepacia* CEP511. CEP511 is a clinical isolate that is susceptible to both trimethoprim and chloramphenicol and is readily transformed (Mahenthiralingam *et al.*, 2000b). A marked deletion of the efflux 5 transporter was introduced into CEP511 via pBAD33. Selection for trimethoprim resistant and chloramphenicol sensitive colonies yielded a double recombination event in which the wild-type transporter was replaced by the deleted gene. Integration of the marked deletion into the host chromosome was verified by PCR amplification of the FRT cassette (Figure 3.4 B).

Once the deletion was returned to the host chromosome, the FRT cassette was excised. The cells were transformed with Flp-producing plasmid pFLP2Cm. After shaking at 30°C for 1 hour, the cells were transferred to 37°C for 1 hour. During the 37°C incubation, the temperature sensitive cI repressor was inactivated, allowing expression of the Flp gene. After incubation, cells containing pFLP2Cm were selected by growth on LB agar with 80 µg/mL chloramphenicol. In order to identify cells that had undergone Flp-mediated excision of the FRT cassette, chloramphenicol resistant colonies were assessed for growth on LB agar with 100 µg/mL trimethoprim. PCR amplification with oligonucleotide primers specific to the FRT cassette was performed to confirm absence of the FRT cassette (Figure 3.4 B). Following excision of the FRT cassette,

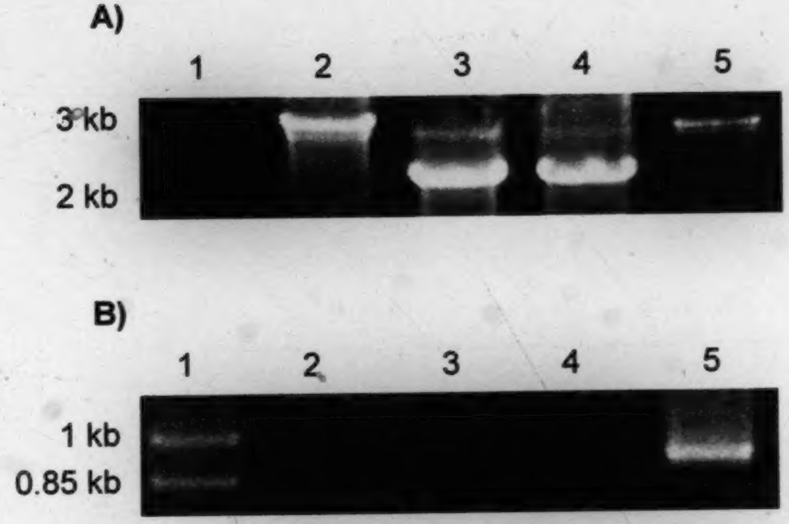

Figure 3.4 PCR confirmation of a markerless deletion in a *B. cenocepacia* CEP511 efflux transporter. Lanes: 1) 1 kb Plus DNA ladder, 2) wild-type CEP511, 3 and 4) CEP511 Δefflux 5 after excision of the FRT cassette, 5) CEP511 Δefflux 5 before excision of the FRT cassette. (A) PCR amplification within the efflux 5 RND transporter using primers Efflux 5.2 2L and 2R (Table 2.3) demonstrates a deletion of 978 bp (in lanes 3 and 4) compared to the wild-type PCR product of ~ 3 kb (lane 2). In lane 5, the efflux 5 PCR product is approximately the same size as the wild-type product. This reflects the simultaneous deletion of 978 bp and insertion of the ~ 900 bp FRT cassette. (B) PCR amplification using primers FRT 1.L and 1.R, which flank the entire FRT cassette (Hoang *et al.*, 1998), verifies that the FRT cassette has been removed from Δefflux 5 strains (lanes 3 and 4) following transformation with pFLP2Cm. As expected, the FRT cassette is amplified from the Δefflux 5 mutant prior to excision of the FRT cassette (lane 5). Wild-type CEP511 (lane 2) was included as a negative control.

pFLP2Cm must be removed from cells. This was accomplished by growth on LB + 5% sucrose plates because cells containing pFLP2Cm, and thus the *sacB* gene, are unable to survive under these conditions. Loss of pFLP2Cm was verified by the inability to grow on 80 μg/mL chloramphenicol and by failure to isolate plasmid DNA. PCR amplification using oligonucleotide primers flanking the deletion was performed to verify the loss of 978 bp from the efflux 5 transporter (Figure 3.4 A).

3.4 Characterization of a *B. cenocepacia* efflux transporter deletion

Having isolated a markerless deletion in the efflux 5 RND transporter gene, we then wanted to compare the mutant to wild-type CEP511 in terms of antibiotic resistance. It was expected that the mutant would show increased sensitivity to certain antimicrobial agents that were substrates of efflux system 5. The minimum inhibitory concentrations (MICs) of 14 antibiotics were determined (Table 3.1). A variety of antimicrobial agents were chosen, including β-lactams, fluoroquinolones, tetracycline, macrolides, aminoglycosides, and other classes. Although there is not necessarily a correlation between homology and substrate range, sensitivity to some *P. aeruginosa* MexEF-OprN substrates, chloramphenicol, fluoroquinolones, and trimethoprim, was determined. For most of the antibiotics tested, there was no difference between wild-type CEP511 and Δefflux 5. Slight differences were observed for some antibiotics. The mutant strain was two times less resistant to novobiocin and two times more resistant to norfloxacin than wild-type CEP511. However, these differences are not necessarily significant as they correspond to only a one or two well difference in the 96 well assay.

Table 3.1 Minimum inhibitory concentrations of various antibiotics against wild-type *B. cenocepacia* CEP511and Δefflux 5. The table below shows the results from one experiment. Susceptibility to ampicillin (AP), carbenicillin (CA), chloramphenicol (CM), erythromycin (ER), fusidic acid (FU), kanamycin (KN), nalidixic acid (NL), norfloxacin (NR), novobiocin (NV), polymyxin B (PB), streptomycin (ST), tetracycline (TC), tobramycin (TB), and trimethoprim (TP) was assessed.

	MIC (µg/mL)											
	AP	ER	FU	KN	NL	NR	NV	PB	ST	TC	TB	TP
CEP511	8000	250	125	4000	4000	125	31.25	4000	>4000	125	2000	7.8
Δefflux 5	8000	250	125	4000	4000	250	15.6	4000	>4000	125	2000	7.8

3.5 Expression of the putative RND efflux pumps in *B. cenocepacia*

One possible explanation for the lack of antibiotic sensitivity in the deletion mutant is that efflux system 5 may not be constitutively expressed in wild-type cells. To address this possibility, expression of the six putative efflux systems was analyzed by RT-PCR. We prepared cDNA from *B. cenocepacia* J2315 grown to mid-logarithmic phase in LB broth. PCR amplification was performed using primers designed within each transporter gene (Figure 3.5). The specificity of the primers was verified by DNA sequencing. Primers within the constantly expressed *recA* gene were used as a positive control. As expected, the *recA* gene was expressed, resulting in a 791 bp band. Efflux system 2 was also expressed, as demonstrated by the 1099 bp product. However, the absence of RT-PCR bands from efflux 1, 3, 4, 5, and 6 specific primers indicates that these efflux systems are not expressed under normal laboratory conditions. There was a lower molecular weight product in the RT-PCR of efflux system 5. The procedure was modified to eliminate this non-specific product. Since *B. cenocepacia* CEP511 was used in the creation of the Δefflux 5 mutant, expression of the RND systems in this strain was also assessed (Figure 3.6). As in J2315, the *recA* positive control was expressed in CEP511. For the most part, none the six putative RND efflux systems were amplified under normal laboratory conditions. However, there may be a faint RT-PCR product corresponding to efflux system 3. To determine if this efflux system is expressed, optimization of the RT-PCR conditions by using various ratios of betaine and DMSO is required. The lack of expression of efflux system 5 in both *B. cenocepacia* strains explains why Δefflux 5 was no different than CEP511 in terms of antibiotic

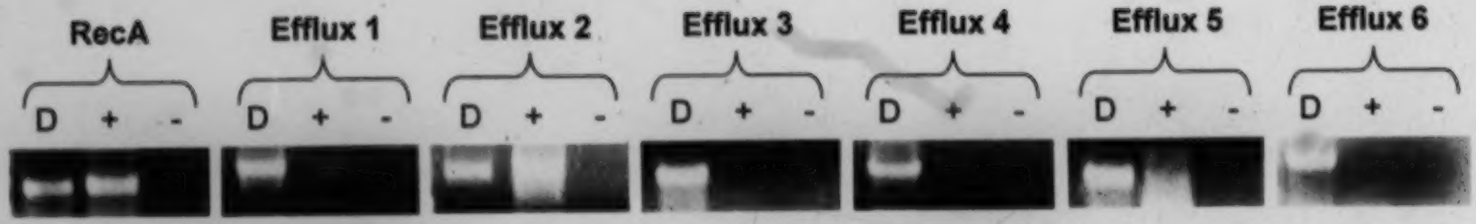

Figure 3.5 Expression of RND efflux pumps in *B. cenocepacia* J2315. RT-PCR amplification was performed on RNA isolated from mid-logarithmic phase cells grown in LB medium. Putative RND efflux transporters 1 – 6 were amplified for 28 cycles with gene-specific primers (+). RT-PCR of the constantly expressed *recA* was performed as a positive control. For each set of oligonucleotide primers, amplification of cDNA synthesized without reverse transcriptase (-) was performed to determine the extent of amplification from contaminating chromosomal DNA. As an additional control, PCR amplification from J2315 chromosomal DNA (D) was carried out to ensure that all reaction reagents and conditions were functioning.

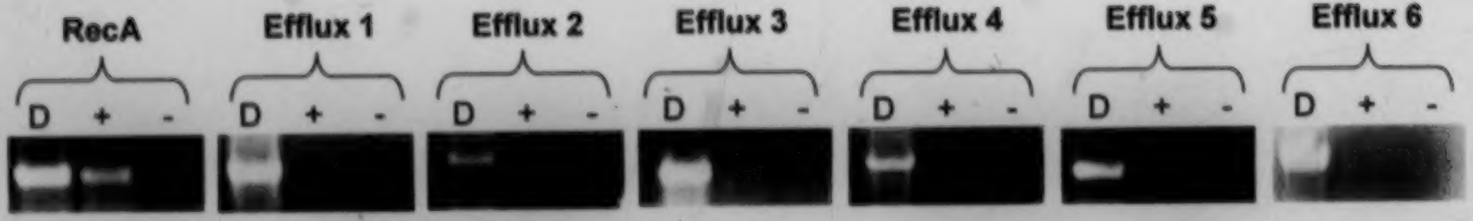

Figure 3.6 Expression of RND efflux pumps in *B. cenocepacia* CEP511. RT-PCR amplification was performed on RNA isolated from mid-logarithmic phase cells grown in LB medium. Putative RND efflux transporters 1 – 6 were amplified for 28 cycles with gene specific primers (+). RT-PCR of the constantly expressed *recA* gene was performed as a positive control. Amplification from cDNA synthesized without reverse transcriptase (-) was included as a negative control for contaminating DNA. PCR amplification from CEP511 chromosomal DNA (D) was performed as a control for PCR conditions and reagents.

susceptibility. An alternate approach was needed to determine the contribution of these efflux systems to *B. cenocepacia* antibiotic resistance.

3.6 Overexpression of efflux 5 structural genes

Overexpression of efflux structural genes from cosmids or plasmids has been associated with increased resistance to antimicrobials for many efflux systems, including *P. aeruginosa mexXY* (Aires *et al.*, 1999), *mexEF-oprN* (Kohler *et al.*, 1997), and *B. cenocepacia ceoAB-opcM* (Nair *et al.*, 2004). A similar approach was attempted for *B. cenocepacia* J2315 efflux system 5. The putative promoter region, as well as the membrane fusion, RND transporter, and outer membrane components, were PCR amplified from J2315 chromosomal DNA. Using *Bam*HI restriction endonuclease sites engineered into the oligonucleotide primers, the structural genes and promoter were cloned into medium copy number plasmids pBBR1MCS and pBBR1MCS-Tp. Two plasmid vectors were chosen because they carry different antibiotic resistance markers (chloramphenicol and trimethoprim, respectively). Using two vectors ensured that increased resistance to either antibiotic could be attributed to overexpression of the efflux system rather than to the vector.

The constructs were electroporated into *B. cenocepacia* CEP511. MICs of various antibiotics were determined for the wild-type, vector control, and overexpression strains (Table 3.2). There was no difference in the MICs of carbenicillin, chloramphenicol, erythromycin, norfloxacin, and trimethoprim other than that due to the vector. A two fold decrease in kanamycin resistance was observed in the pBBR1MCS overexpression strain. A two fold increase in tetracycline resistance was seen in the

Table 3.2 Overexpression of efflux 5 structural genes in *B. cenocepacia* CEP511. The efflux 5 structural genes were cloned into pBBR1MCS and pBBR1MCS-Tp. The minimum inhibitory concentration (MIC) of various antibiotics on wild-type, vector controls, and overexpression strains was compared. The values below are an average of two replicates, with the exception of carbenicillin. Antibiotics carbenicillin (CA), chloramphenicol (CM), erythromycin (ER), kanamycin (KN), norfloxacin (NR), tetracycline (TC), and trimethoprim (TP) were used.

	MIC (µg/mL)						
	CA	**CM**	**ER**	**KN**	**NR**	**TC**	**TP**
CEP511	4000	31.25	125	4000	250	62.5	7.8
+ pBBR1MCS	4000	250	125	4000	250	62.5	7.8
+ pBBR1MCS + efflux 5	4000	250	125	4000	250	125	7.8
+ pBBR1MCS-Tp	4000	31.25	125	4000	250	62.5	250
+ pBBR1MCS-Tp + efflux 5	4000	31.25	125	2000	250	62.5	250

pBBR1MCS overexpression strain. Since these differences were not seen in both pBBR1MCS and pBBR1MCS-Tp overexpression strains, they are not significant. A similar experiment performed in *E. coli* DH5α also showed no significant difference in resistance between wild-type strains and strains overexpressing efflux system 5 (data not shown). Introduction of the efflux 5 structural genes in *B. cenocepacia* CEP511 and in *E. coli* did not increase resistance to any antimicrobial agents tested.

To verify that the efflux genes were expressed from the plasmid system, RT-PCR experiments were performed using primers within the efflux 5 RND transporter gene. In order to eliminate possible expression of the chromosomal copy, the plasmids containing the efflux 5 structural genes were electroporated into Δefflux 5. RNA isolated from both Δefflux 5 and Δefflux 5 containing the plasmid-borne structural genes was subjected to RT-PCR using efflux 5 transporter specific primers. Results of the RT-PCR experiment show that, at the RNA level, the efflux 5 genes are expressed from the plasmid vectors (Figure 3.7). Although the 584 bp band in the minus reverse transcriptase control indicated that some amplification is the result of DNA contamination, the band from the reaction with reverse transcriptase was noticeably brighter. Therefore, there is efflux system 5 mRNA present.

3.7 Overexpression of a putative regulator of efflux system 5

Another approach to increasing the levels of an efflux pump is to overexpress its corresponding activator. Located near the genes for *B. cenocepacia* efflux system 5 is a putative LysR-type regulatory gene. LysR-type proteins are usually transcription activators that require an inducing molecule for their function (Schell, 1993). However,

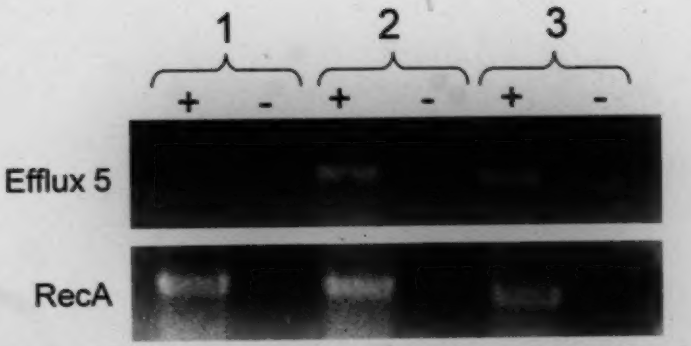

Figure 3.7 Expression of efflux 5 structural genes from pBBR1MCS and pBBR1MCS-Tp. In order to confirm expression of the efflux 5 structural genes from the overexpression vectors, RT-PCR experiments were performed. RNA was isolated from mid-logarithmic phase cells of (1) Δefflux 5, (2) Δefflux 5 plus efflux 5 structural genes on pBBR1MCS, and (3) Δefflux 5 plus efflux 5 structural genes on pBBR1MCS-Tp. First strand synthesis and 28 cycles of PCR amplification were performed using gene specific primers within the efflux 5 RND transporter (+) and within *recA* (positive control). As a control, amplification was performed using cDNA synthesized without reverse transcriptase (-).

overexpression of *P. aeruginosa mexT*, encoding a LysR-type regulator, from a plasmid is sufficient to induce expression of the *mexEF-oprN* efflux operon and increase antibiotic resistance (Kohler *et al.*, 1997; Kohler *et al.*, 1999). As such, it was hoped that overexpression of the putative regulator of *B. cenocepacia* efflux system 5 would have a similar effect.

The putative LysR-type activator was PCR amplified without its promoter and cloned into the pBBR1MCS vector by means of *Pst*I and *Xba*I sites engineered into the oligonucleotide primers. The resulting construct was electroporated into CEP511 wild-type and Δefflux 5 strains and the MICs of various antibiotics were determined (Table 3.3). Overexpression of the activator did not increase resistance to any of the antibiotics tested. The observed increase in chloramphenicol resistance is due to the presence of a chloramphenicol resistance marker on pBBR1MCS. The two fold increase in resistance to fusidic acid shown by Δefflux 5 is not significant. However, expression of the LysR-type activator from pBBR1MCS has not been verified.

3.8 Expression of *B. cenocepacia* efflux pumps in response to chloramphenicol and salicylate

Given that none of the putative efflux systems were expressed in CEP511, we wanted to find conditions that may turn on efflux pump expression in *B. cenocepacia*. Identifying inducers of efflux pump expression would provide clues to the possible function of the efflux system. Also, being able to induce expression of an efflux system would allow us to examine the phenotype of efflux pump deletion mutants. Both salicylate and chloramphenicol have been identified as inducers of the *ceoAB-opcM*

Table 3.3 The effect of overexpression of the putative LysR-type regulator of efflux system 5 on antibiotic resistance. The putative regulatory gene was cloned into pBBR1MCS and expressed in wild-type CEP511 and Δefflux 5. The data below are the results of one experiment. Minimum inhibitory concentrations (MICs) of chloramphenicol (CM), erythromycin (ER), fusidic acid (FU), norfloxacin (NR), polymyxin B (PB), sulfamethoxaxole (SX), tetracycline (TC), and trimethoprim (TP) were determined.

	MIC (μg/mL)						
	CM	**ER**	**FU**	**PB**	**SX**	**TC**	**TP**
CEP511	15.6	125	125	>2000	125	62.5	15.6
+ pBBR1MCS	250	125	125	>2000	125	62.5	15.6
+ pBBR1MCS + regulator	250	125	125	>2000	125	62.5	15.6
Δ efflux 5	15.6	125	250	>2000	125	62.5	15.6
+ pBBR1MCS	250	125	125	>2000	125	62.5	15.6
+ pBBR1MCS + regulator	250	125	125	>2000	125	62.5	15.6

efflux system in *B. cenocepacia* PC121 (Nair *et al.*, 2004). To determine if salicylate and chloramphenicol were inducers of any of the six putative *B. cenocepacia* J2315 efflux systems, RT-PCR experiments were performed (Figure 3.8). Cells were grown to mid-logarithmic phase in LB medium with or without 1 mM salicylate or 5 µg/mL chloramphenicol. The expression of the *recA* positive control was not changed by the presence of salicylate or chloramphenicol. Efflux systems 1, 3, 4, 5, and 6 were not expressed under any of the conditions examined. Although efflux system 6 is a close homologue of *ceoAB-opcM*, its expression was not induced by chloramphenicol or salicylate. This could be a strain dependent difference. *B. cenocepacia* PC121, the strain used by Nair *et al.* (2004), is a salicylate producing clinical isolate in which antibiotic resistance is known to increase in low iron environments. *B. cenocepacia* J2315 efflux system 2 was expressed in all three conditions but seemed to be expressed at a higher level in the presence of 1 mM salicylate. In order to verify that efflux system 2 is induced by salicylate, the experiment was repeated using a range of salicylate concentrations (Figure 3.9). This time, there was no noticeable difference in expression levels between the 0 and 1 mM salicylate conditions. The RT-PCR product in the 2.5 mM and 5 mM conditions were only marginally brighter than that in 0 mM salicylate. This indicates that efflux system 2 is not induced strongly by salicylate.

3.9 Expression of *B. cenocepacia* efflux pumps in response to growth phase

Expression of some RND efflux systems is linked to growth. Expression of *E. coli* multidrug transporter *acrAB* increases in slow-growing cells (Rand *et al.*, 2002). In *P. aeruginosa*, expression of the *mexAB-oprM* multidrug efflux system increases during

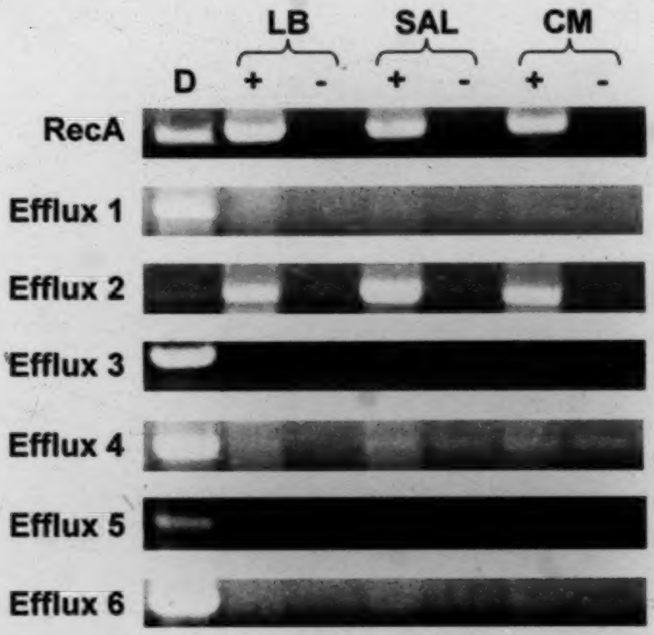

Figure 3.8 Effect of salicylate and chloramphenicol on efflux pump expression in *B. cenocepacia* J2315. RT-PCR amplification of the six putative RND transporter genes (+) was performed using RNA from J2315 cells grown under three conditions: cells grown in LB medium (LB), cells grown in LB with 1 mM salicylate (SAL), and cells grown in LB with 5 µg/mL chloramphenicol (CM). Amplification using *recA* primers was performed as a loading control. A negative control was performed, amplifying cDNA that was synthesized without reverse transcriptase (-). As an additional control, PCR amplification from J2315 chromosomal DNA (D) was carried out to ensure that all reaction reagents and conditions were functioning.

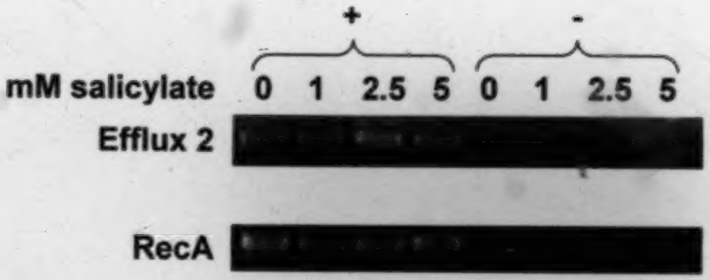

Figure 3.9 Effect of salicylate on expression of efflux system 2 in *B. cenocepacia* J2315. J2315 was grown in the presence of various amounts of salicylate. RT-PCR amplification for 28 cycles was performed using oligonucleotide primers specific to the RND transporter of efflux system 2 (+). Amplification using *recA* primers was performed as a loading control. A minus reverse transcriptase control (-) was performed to assess amplification from contaminating DNA.

late logarithmic and stationary phase growth (Evans and Poole, 1999; Maseda *et al.*, 2004). It seemed likely that expression of some putative *B. cenocepacia* RND efflux systems might respond to growth phase in the same manner. RT-PCR experiments were performed using RNA isolated from J2315 cells grown to late-logarithmic phase and stationary phase (Figure 3.10). MexAB-like efflux system 2 was expressed during late-logarithmic phase but not during stationary phase. The other five putative RND efflux systems were not expressed in late-logarithmic or stationary phase. Following amplification of cDNA synthesized from stationary phase RNA preparations, there was considerable DNA contamination due to difficulties isolating pure RNA from these cells. A similar experiment was performed in *B. cenocepacia* CEP511 (Figure 3.11). In this strain, efflux system 2, which was not amplified from mid-logarithmic phase cells (Figure 3.6), was expressed in late-logarithmic and stationary phase cells. However, efflux systems 1, 3, 4, 5, and 6 were not expressed under any conditions tested.

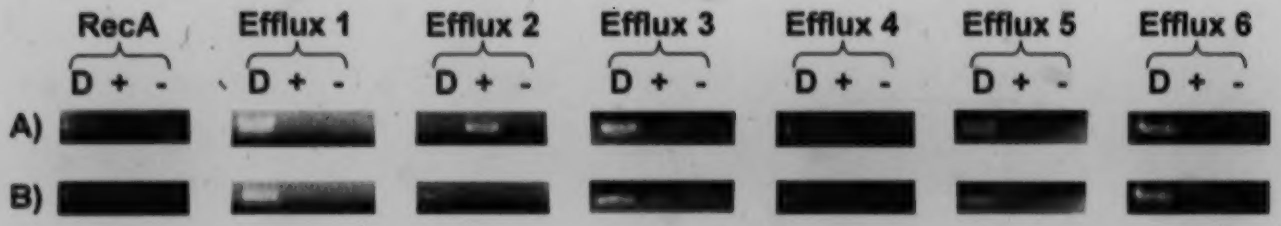

Figure 3.10 Expression of *B. cenocepacia* J2315 efflux systems during late-logarithmic and stationary phase growth. RT-PCR amplification of the six putative RND efflux pumps was performed using 0.75 μg total RNA from (A) cells grown to late-logarithmic phase (OD_{600} = 1.2) or (B) cells grown to stationary phase (growth overnight). PCR amplification (28 cycles) was performed using primers specific to the six putative RND transporter genes (+). Amplification of *recA* was done as a loading control. A minus reverse transcriptase control (-) was used to determine amplification of contaminating DNA. To control for PCR reagents and conditions, amplification from J2315 chromosomal DNA was performed (D).

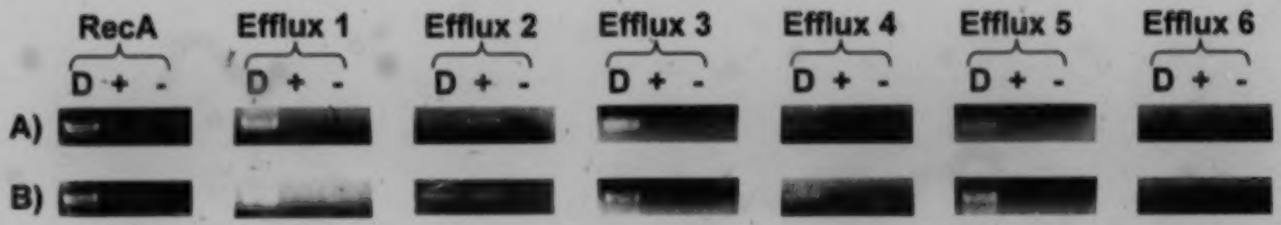

Figure 3.11 Expression of *B. cenocepacia* CEP511 efflux systems during late-logarithmic and stationary phase growth. RT-PCR amplification of the six putative RND efflux pumps was performed using 0.75 µg total RNA from (A) cells grown to late-logarithmic phase (OD_{600} = 1.2) or (B) cells grown to stationary phase (growth overnight). PCR amplification (28 cycles) was performed using primers specific to the six putative RND transporter genes (+). Amplification of *recA* was done as a loading control. A minus reverse transcriptase control (-) was used to determine amplification of contaminating DNA. To control for PCR reagents and conditions, amplification from CEP511 chromosomal DNA was performed (D).

Chapter 4: Discussion

4. DISCUSSION

Antibiotic resistance in the *B. cepacia* complex poses a considerable barrier to the treatment of opportunistic infections. In many cases, triple antibiotic combinations may be required for eradication of the *B. cepacia* complex from CF patients (Aaron *et al.*, 2000; Burns and Saiman, 1999). We hypothesize that this extensive resistance is largely due to uncharacterized multidrug efflux pumps that can bind antibiotics and toxic compounds and export them from the cell. Widespread antibiotic resistance is also a considerable challenge in performing mutational analysis of the *B. cepacia* complex. As such, the goal of this research project was two-fold: to determine the contribution of multidrug efflux pumps to antibiotic resistance in the *B. cepacia* complex, and to develop a Flp/FRT system by which markerless mutations can be created.

4.1 Identification and characterization of multidrug efflux systems in the *B. cepacia* complex

Multidrug efflux pumps have emerged as a major problem in the treatment of pathogenic bacteria. A great deal of research has focused on the role of such systems in *E. coli* and *P. aeruginosa*. However, knowledge of multidrug efflux pumps in the *B. cepacia* complex is limited. Currently, only three systems have been identified in members of the *B. cepacia* complex. An RND efflux system, *ceoAB-opcM*, contributes to chloramphenicol, ciprofloxacin, and trimethoprim resistance in *B. cenocepacia* (Burns *et al.*, 1996). BcrA, an MFS pump from *B. cenocepacia*, increases resistance to tetracycline and nalidixic acid in *E. coli*, suggesting it may play a similar role in the *B. cepacia* complex (Wigfield *et al.*, 2002). The third efflux system is a MATE family transporter that confers resistance to polymyxin B in *B. vietnamiensis* (Fehlner-Gardiner and Valvano, 2002). However, these efflux systems do not account for the full extent of antibiotic resistance in the *B. cepacia* complex. The recent completion of the *B. cenocepacia* J2315 genome sequence (http://www.sanger.ac.uk/Projects/B_cenocepacia/) suggested a genomics approach to identifying additional RND-type systems in the *B. cepacia* complex. By searching the genome sequence of *B. cenocepacia* J2315 with DNA sequences from known efflux pumps in related bacteria, we have identified six putative resistance nodulation cell division (RND) efflux pumps (Figure 3.1). Five of these pumps had considerable homology to multidrug transporters of *P. aeruginosa* and therefore are possible contributors to the high intrinsic resistance of *B. cenocepacia*. The remaining RND-type system is homologous to a heavy metal cation transporter. It is not unusual for an organism to possess multiple efflux systems. The genome of *E. coli* contains at least 37 established and putative drug transporters, including seven of the RND family (Nishino and Yamaguchi, 2001b). Likewise, the *P. aeruginosa* genome contains 12 potential RND-type efflux systems, only six of which have been characterized (Stover *et al.*, 2000).

In terms of genetic organization, the *B. cenocepacia* efflux clusters typically consist of an inner membrane transporter, a membrane fusion subunit, and an outer membrane protein channel. The only exception is efflux system 3, which is an isolated RND transporter. In general, their genetic arrangement is similar to *P. aeruginosa* efflux operons in which the membrane fusion and RND components are always encoded together. Genes encoding regulators and outer membrane proteins are often, but not always present (Schweizer, 2003). Some *P. aeruginosa* efflux operons contain additional genes, such as an extra RND transporter or a membrane protein required for drug efflux

(Aendekerk *et al.*, 2002; Schweizer, 2003). This was not observed for the six putative RND systems in *B. cenocepacia*. Efflux system 3, the independent transporter gene, may be similar to *E. coli* transporter AcrD. The gene encoding AcrD is not found with corresponding membrane fusion and outer membrane proteins. However, AcrD is a functional efflux pump that exports aminoglycosides, bile salts, and novobiocin. It was recently discovered that AcrD works in conjunction with non-cognate membrane fusion protein AcrA and outer membrane protein TolC (Elkins and Nikaido, 2002; Rosenberg *et al.*, 2000). Therefore, *B. cenocepacia* efflux system 3 may utilize the membrane fusion protein and outer membrane protein from another RND system.

Although organisms may contain multiple RND systems, not all contribute to intrinsic resistance. For example, the constitutively expressed *E.coli* AcrAB-TolC efflux pump predominates in terms of resistance to commonly used antimicrobial agents (Nikaido, 1996). Similarly, of the six characterized *P. aeruginosa* RND-type systems, only MexAB-OprM (Li *et al.*, 1995; Poole *et al.*, 1993a; Poole *et al.*, 1993b), MexXY (Aires *et al.*, 1999), and MexGHI (Aendekerk *et al.*, 2002) are involved in intrinsic resistance under normal laboratory conditions. MexCD-OprJ (Poole *et al.*, 1996a; Srikumar *et al.*, 1997), MexEF-OprN (Kohler *et al.*, 1997), and MexJK (Chuanchuen *et al.*, 2002) are not expressed to a significant extent in wild-type cells.

In order to determine which of the putative RND systems were expressed in *B. cenocepacia*, RT-PCR analysis was performed using primers specific to the transporter components. As observed in *E. coli* and *P. aeruginosa*, few of the *B. cenocepacia* efflux systems are expressed under normal laboratory conditions in mid-logarithmic phase cells. In J2315, the source of the efflux sequences, only efflux system 2 was expressed.

Generally, the constitutively expressed efflux pumps, such as AcrAB of *E. coli* and MexAB-OprM of *P. aeruginosa*, are the most important in terms of intrinsic antibiotic resistance. Interestingly, the closest homologue of efflux system 2 was the MexAB-OprM RND system. This evidence strongly suggests that efflux system 2 may be of great importance to the intrinsic resistance of wild-type *B. cenocepacia*. Although efflux system 2 was expressed in *B. cenocepacia* J2315 during mid-logarithmic phase growth, it was not expressed in another *B. cenocepacia strain*, CEP511. This is an intriguing result as there are differences in the antibiotic susceptibilities of these two strains. J2135 is more resistant than CEP511 to ceftazidime, tobramycin, tetracycline, and trimethoprim (Nzula *et al.*, 2002). Our studies indicate that J2315 is also more resistant than CEP511 to chloramphenicol. Perhaps the difference in resistance to these antibiotics can be explained by the action of efflux system 2. Further experiments will be necessary to determine if these antibiotics are in fact efflux 2 substrates.

It was somewhat surprising that efflux system 6 was not expressed in either *B. cenocepacia* J2315 or CEP511. According to RT-PCR results, the *ceoAB-opcM* efflux system is constitutively expressed in two *B. cenocepacia* clinical isolates, K61-3 and PC121 (Nair *et al.*, 2004). Since efflux system 6 is 93% identical to the CeoB transporter at the protein level, we expected that efflux system 6 would be expressed in J2315 and CEP511, which are also *B. cenocepacia* clinical isolates (Mahenthiralingam *et al.*, 2000b). Nair *et al.* report that CEP511 does not contain the membrane fusion and outer membrane protein components of the *ceoAB-opcM* operon (Nair *et al.*, 2004), which could account for the lack of expression in this strain. However, their study relied on PCR amplification of the *ceo* components, using only a single pair of primers for each gene. In this case, a difference of only a few nucleotides between strains could completely abolish PCR amplification. Southern hybridization may have provided more meaningful results. Another possible explanation for the lack of efflux 6 expression could lie in the natural function of the *ceoAB-opcM* efflux system. Based on the observation that CeoAB-OpcM is able to extrude salicylate, an important siderophore and signal molecule in plant pathogenesis, Nair *et al.* suggest that salicylate may be the true natural substrate for the pump (Nair *et al.*, 2004). If this is true, it could be that only salicylate secreting strains of the *B. cepacia* complex would express the *ceoAB-opcM* (or efflux system 6). In support of this theory, K61-3 and PC121 constitutively express the *ceoAB-opcM* efflux operon and secrete salicylate (Nair *et al.*, 2004). On the other hand, CEP511 does not secrete salicylate (Nair *et al.*, 2004) and, in our study, was shown not to express the CeoB-like efflux system 6. However, some strains are able to secrete salicylate even though some components of the *ceoAB-opcM* system could not be amplified from their genomes (Nair *et al.*, 2004).

Although five of the efflux systems were not expressed, they may still be clinically important. Multidrug resistant bacteria are often selected for *in vivo* during antimicrobial therapy. Many of these resistant bacteria overexpress multidrug efflux pumps. *P. aeruginosa nalB* mutants, overexpressing MexAB-OprM, have been found among resistant clinical isolates (Bert and Lambert-Zechovsky, 1996; Li *et al.*, 1994a; Li *et al.*, 1994b; Ziha-Zarifi *et al.*, 1999). Likewise, the MexCD-OprJ and MexEF-OprN efflux systems are expressed in clinical *nfxB* (Jakics *et al.*, 1992; Jalal and Wretlind, 1998) and *nfxC* (Beinlich *et al.*, 2001) mutants respectively. Increased expression of efflux systems in clinical multidrug resistant strains have also been reported in many other Gram-negative organisms (Li and Nikaido, 2004). Therefore, it is possible that multidrug resistant *B. cepacia* complex strains overexpressing one or more of the RND systems could be selected for during antimicrobial therapy of CF patients.

Alternatively, the efflux systems may each play a unique role in the cell. In this case, the pumps may only be expressed in response to certain inducing molecules or environmental conditions. For example, expression of some RND efflux systems is linked to growth phase. Expression of *E. coli* multidrug transporter *acrAB* is inversely related to growth rate (Rand *et al.*, 2002). Similarly, in *P. aeruginosa*, expression of the *mexAB-oprM* multidrug efflux system increases during late-logarithmic and stationary phase growth (Evans and Poole, 1999; Maseda *et al.*, 2004; Sanchez *et al.*, 2002b). Several links connect expression of efflux pumps with quorum sensing. The *P. aeruginosa mexGHI-opmD* operon is induced by acyl homoserine lactones (Whiteley *et al.*, 1999) and mutation of *mexI* or *opmD* reduces the presence of quorum sensing regulated phenotypes (Aendekerk *et al.*, 2002). Loss of the LuxR-type quorum sensing regulator VsqR decreases expression of the MexEF-OprN efflux system (Juhas *et al.*, 2004).

We conducted RT-PCR experiments on *B. cenocepacia* strains J2315 and CEP511 to determine the extent of efflux pump expression during late-logarithmic and stationary phase growth. Efflux system 2 was expressed in late-logarithmic phase cells of J2315 (Figure 3.10). In CEP511, efflux system 2 was expressed strongly during late-logarithmic phase and to a lesser extent during stationary phase (Figure 3.11). Unfortunately, no comparisons can be made with the RT-PCR results from mid-logarithmic phase cells (Figures 3.5 and 3.6) since the growth medium and amount of

RNA template were not kept constant. Quantitative RT-PCR should be performed on RNA isolated from all three growth phases, in the same medium, in order to validate growth phase regulation of efflux system 2. The remaining five RND systems were not expressed in late-logarithmic or stationary phase cultures of J2315 or CEP511 (Figures 3.10 and 3.11).

Growth in the presence of salicylate is another factor that induces antibiotic resistance in many organisms, including *Klebsiella pneumoniae* (Domenico *et al.*, 1990), *Mycobacterium tubersulosis* (Schaller *et al.*, 2002), and *Staphylococcus aureus* (Gustafson *et al.*, 1999; Price *et al.*, 1999). In *E. coli*, salicylate interacts directly with MarR to alleviate repression of the *marRAB* operon and thus increase antibiotic resistance (Alekshun *et al.*, 2001; Cohen *et al.*, 1993). In *P. aeruginosa*, the presence of salicylate causes increased resistance to imipenem by decreasing the production of OprD, the outer membrane channel responsible for imipenem influx (Masuda *et al.*, 1995; Ochs *et al.*, 1999; Sumita and Fukasawa, 1993). Salicylate also increases antimicrobial resistance in *B. multivorans* 249-2 (Burns and Clark, 1992) and induces expression of the CeoAB-OpcM efflux system in *B. cenocepacia* PC121 (Nair *et al.*, 2004). Since salicylate is often associated with increased antimicrobial resistance, we tested its effect on the expression of the *B. cenocepacia* J2315 RND systems. RT-PCR with primers specific to each RND transporter was performed on RNA isolated from cells grown in the presence of 1 mM salicylate. Growth in the presence of salicylate did not affect the expression of most *B. cenocepacia* J2315 efflux pumps. Efflux systems 1, 3, 4, 5, and 6 were not expressed, regardless of the presence of salicylate. Initially, it appeared that expression of efflux system 2 was increased slightly by 1 mM salicylate (Figure 3.8). However, when a larger range of salicylate concentrations was tested, 1 mM salicylate did not increase efflux 2 expression (Figure 3.9). There appeared to be a marginal increase in the expression of efflux 2 in the presence of 2.5 mM and 5.0 mM salicylate, however, the results are not conclusive. Quantitative real-time PCR must be performed to accurately detect a difference in expression of efflux system 2.

Chloramphenicol is also an inducer of the *ceoAB-opcM* efflux system in *B. cenocepacia* PC121 (Nair *et al.*, 2004). Accordingly, we performed RT-PCR to determine if the presence of 5 μg/mL chloramphenicol increased expression of the *B. cenocepacia* J2315 RND systems (Figure 3.8). Results were similar to those observed in the presence of salicylate. No increase in expression was detected for any of the putative efflux systems in the presence of chloramphenicol. There are many other possibilities for efflux pump inducers that have yet to be tested. For example, expression of *P. aeruginosa mexCD-oprJ* increases in response to some of its substrates, tetraphenylphosphonium, ethidium bromide, rhodamine 6G, acriflavine, and disinfectants (Morita *et al.*, 2003). Similarly, MexXY is induced by pump substrates tetracycline, erythromycin, and gentamicin (Masuda *et al.*, 2000b). Once the substrates of the *B. cenocepacia* efflux pumps are determined, we will have a better idea of potential inducers to test with RT-PCR.

Several strategies were employed in an attempt to determine the substrates of each *B. cenocepacia* RND efflux system. Much of the work focused on efflux system 5. First, a markerless deletion was created within the efflux 5 RND transporter gene of *B. cenocepacia* CEP511 (Δefflux 5). The deletion was verified by PCR amplification with primers flanking the deletion (Figure 3.4). However, MIC assays revealed no difference in the susceptibility of wild-type CEP511 and Δefflux 5 to 14 antibiotics (Table 3.1). The lack of phenotype in Δefflux 5 was later explained when RT-PCR results revealed that efflux system 5 is not expressed in CEP511 under normal growth conditions (Figure 3.6). In fact, all of the efflux systems, except efflux system 2, were not expressed in *B. cenocepacia* J2315 or CEP511 (Figures 3.5 and 3.6). Therefore, the creation of deletions in wild-type cells is not an effective approach for the study of these systems. This problem has been encountered by other laboratories. Inactivation of *E. coli* efflux pumps AcrEF, YhiUV, and MdtABCD does not affect drug susceptibility (Baranova and Nikaido, 2002; Nishino and Yamaguchi, 2001b; Sulavik *et al.*, 2001). Likewise, mutation of *mexE*, the membrane fusion component of the MexEF-OprN efflux system, does not change the resistance profile of wild-type *P. aeruginosa* (Kohler *et al.*, 1997). Given that efflux system 2 is expressed at some point in *B. cenocepacia* J2315 and CEP511, a deletion of this transporter may produce a change in the antibiotic resistance profile.

In some cases, the introduction of extraneous copies of an efflux system on plasmids or cosmids confers antimicrobial resistance. For example, introduction of the *ceoAB-opcM* operon into *B. cenocepacia* CEP511 increases resistance to ciprofloxacin, chloramphenicol, and trimethoprim (Nair *et al.*, 2004). Similarly, expression of the *mexXY* genes from a plasmid increased the resistance of wild-type *P. aeruginosa* to MexXY substrates (Aires *et al.*, 1999). Since most of the *B. cenocepacia* efflux systems are not constitutively expressed, the only way to determine potential substrates would be to overexpress the pumps and look for a corresponding increase in resistance to certain antibiotics. Therefore, we cloned the efflux 5 structural genes (membrane fusion protein, RND transporter, and outer membrane protein) along with the putative promoter region into the broad host range vectors pBBR1MCS and pBBR1MCS-Tp. When the resulting constructs were introduced into *B. cenocepacia* CEP511, there was no change in antimicrobial susceptibility (Table 3.2). There are many possible explanations as to why the overexpression of efflux 5 structural genes did not confer antibiotic resistance. RT-PCR analysis confirmed that the structural genes were expressed from the plasmid vectors at the RNA level (Figure 3.7). However, we did not determine whether this increase in mRNA correlated to an increase in protein levels. This could be tested by running SDS-PAGE gels and looking for the presence of three additional protein bands corresponding to the sizes of the efflux 5 gene products. Alternatively, the addition of a marker, such as a His-tag, into one of the open reading frames could be used to verify expression at the protein level. It is also possible that mutations could have been introduced during PCR amplification and cloning of the efflux genes. Although a proofreading polymerase was used during PCR to avoid such a problem, the clone should be sequenced to verify that no muations were introduced. Another explanation is that the overexpression did function as expected, but none of the seven antibiotics tested were substrates for efflux system 5.

Efflux systems can also be overexpressed indirectly by mutating the corresponding regulator. Deletion of *P. aeruginosa* TetR family repressors *mexR* and *mexZ* increase expression of *mexAB-oprM* and *mexXY* respectively (Poole *et al.*, 1996b; Westbrock-Wadman *et al.*, 1999). Overexpression of the *mexAB-oprM*, *mexCD-oprJ*, and *mexJK* efflux systems often results from mutations in the respective regulators (Chuanchuen *et al.*, 2002; Poole *et al.*, 1996a; Saito *et al.*, 1999; Srikumar *et al.*, 2000;

Ziha-Zarifi *et al.*, 1999). Therefore, we attempted to overexpress efflux system 5 by increasing levels of the putative LysR-type activator. The regulatory gene was cloned into pBBR1MCS and introduced into *B. cenocepacia* CEP511 and Δefflux 5. Unfortunately, no change in the resistance profile was observed (Table 3.3). It is possible that none of the tested antibiotics are substrates of efflux system 5. To address another potential problem, RT-PCR analysis should be performed to determine if the regulator is expressed and if the presence of the plasmid-encoded regulator produces the expected increase in efflux 5 expression. DNA sequencing verified that no mutations were introduced during PCR amplification. However, in many wild-type *P. aeruginosa* strains, the gene encoding LysR-type regulator MexT contains inactivating mutations preventing expression of *mexEF-oprN* (Maseda *et al.*, 2000). Since *mexEF-oprN* is the closest *P. aeruginosa* homologue of efflux system 5, it is possible that similar inactivating mutations are found in the efflux 5 regulator. Another possibility is that the efflux 5 regulator, like many LysR-type proteins, may require an effector molecule in order to activate gene expression (Schell, 1993). However, overexpression of *P. aeruginosa mexT*, encoding a LysR-type regulator, from a plasmid is sufficient to induce expression of the *mexEF-oprN* efflux operon and increase antibiotic resistance (Kohler *et al.*, 1997; Kohler *et al.*, 1999).

Similar attempts could be made to interfere with the regulators of the other RND efflux systems. Efflux system 1 and 2 are both located near genes encoding putative AraC-type proteins (Figure 3.1). AraC family members are usually transcriptional activators (Gallegos *et al.*, 1993). MarA, SoxS, and Rob are all AraC-type global activators known to positively regulate the *E. coli* AcrAB RND efflux system (Ma *et al.*, 1996; Martin *et al.*, 1996). Another AraC regulator, MtrA, activates expression of the *Neisseria gonorrhoeae mtrCDE* efflux system (Rouquette *et al.*, 1999). Therefore, efflux systems 1 and 2 may also be positively regulated by the AraC homologs. Like efflux system 5, efflux system 6 is also found near a putative LysR-type activator. If efflux systems 1, 2, 5, and 6 are indeed positively regulated, then overexpression of the activator genes could lead to increased resistance to substrate antibiotics. On the other hand, the gene immediately upstream and in the opposite orientation of efflux system 2 encodes a putative TetR-family repressor. The predicted amino acid sequence of this regulator was similar to *E. coli* AcrR, which negatively regulates expression of *acrAB* (Ma *et al.*, 1996). In this case, deletion of the putative efflux 2 repressor may cause an increase in expression of efflux system 2 and, therefore, an increase in antibiotic resistance.

Although the original goal of characterizing the putative *B. cenocepacia* RND systems in terms of efflux substrates was not achieved, valuable information was obtained that will facilitate future work in this area. Six putative RND systems were identified in the *B. cenocepacia* J2315 genome, five of which showed homology to *P. aeruginosa* multidrug transporters. One of these systems, efflux 2, was similar to MexAB-OprM and was shown to be expressed in strains J2315 and CEP511 at various stages of growth. Therefore, this efflux pump is likely important to the intrinsic resistance of *B. cenocepacia*. Accordingly, efflux system 2 should be the focus of future research. Quantitative real-time PCR will verify induction of efflux 2 expression in response to growth phase and salicylate. The substrate range of efflux 2 could be determined by deletion analysis or overexpression of this system by deleting its putative repressor. Additionally, deletion analysis of the RND efflux systems was shown to be ineffective, as most of the pumps were not constitutively expressed. Overexpression of the efflux systems, either directly or indirectly, should prove more useful in determining the contribution of RND systems to the resistance of *B. cenocepacia*. In this way, substrates of each pump can be identified and their relative ability to induce efflux pump expression can be assessed.

4.2 Development of a Flp/FRT system for use in the *B. cepacia* complex

Flp/FRT systems have proven useful in the creation of markerless mutations of *P. aeruginosa pabC* (Hoang *et al.*, 1998), *mexAB-oprM* (Schweizer, 1998), and *mexXY* (Morita *et al.*, 2001a). In fact Flp/FRT systems have been used to create *P. aeruginosa* strains that lack all four major *mex* operons (Morita *et al.*, 2001b). During this study, we developed the first Flp/FRT system for use in the *B. cepacia* complex. In this system, a gene of interest is cloned into pBAD33, chosen because it does not replicate in members of the *B. cepacia* complex. A deletion is made using appropriate restriction endonucleases and the FRT cassette, containing a trimethoprim resistance marker flanked by two Flp recombinase target sites, replaces the deleted region. The construct is electroporated into a *B. cepacia* complex strain, where it can undergo homologous recombination with the host chromosome. A double recombination event occurs, identified by trimethoprim resistance (from the FRT cassette) and chloramphenicol sensitivity (from loss of pBAD33), replacing the wild-type copy with the marked deletion. At this point, the Flp-producing plasmid pFLP2Cm is introduced by electroporation. Induction of the flippase gene is obtained by incubation at 37°C, resulting in site-specific recombination between the two Flp recombinase targets to excise the FRT cassette. Chloramphenicol resistant (containing pFLP2Cm), trimethoprim sensitive (having lost the FRT cassette) colonies are identified. These cells are then subjected to growth on LB + 5% sucrose, selecting for loss of the *sacB* containing pFLP2Cm. Deletion of the gene of interest and presence or absence of the FRT cassette can be verified by PCR amplification. This system was used successfully for the creation of a CEP511 deletion mutant of the efflux system 5 RND transporter.

While applying the Flp/FRT system, it became apparent that some members of the *B. cepacia* complex are more amenable to mutational analysis. Although the genome sequence of *B. cenocepacia* J2315 is available, this strain proved too difficult for use in laboratory studies as a result of high intrinsic resistance and a negligible transformation rate (Mahenthiralingam *et al.*, 2000b). The strain CEP511 proved much more useful. CEP511 differs from J2315 in that it lacks the *cblA* gene encoding the cable pilus (Mahenthiralingam *et al.*, 2000b). *B. cenocepacia* is divided into two lineages based on the sequence of *recA*. CEP511 belongs to the genomovar IIIB lineage while J2315 belongs to the genomovar IIIA lineage (Mahenthiralingam *et al.*, 2000a). Like J2315, CEP511 is a CF epidemic strain and contains the *B. cepacia* epidemic strain marker (BCESM). Its high transformation rate and susceptibility to trimethoprim and chloramphenicol are tremendous advantages in creating mutations with the Flp/FRT system (Mahenthiralingam *et al.*, 2000b). For the purpose of this study, the gene sequences of CEP511 and J2315 were similar enough that the same oligonoucleotide primers were effective for amplification of RND transporters and *recA* from both strains.

Although CEP511 is a useful lab strain, the applicability of the Flp/FRT system could be extended. By creating additional FRT cassettes and Flp-producing plasmids with different antibiotic markers, the Flp/FRT system could also be used in other members of the *B. cenocepacia* complex. For example, selection for chloramphenicol resistance was problematic in *B. cenocepacia* K56-2 and *B. vietnamiensis* DB01 due to inducible resistance.

The Flp/FRT system developed in this study should prove extremely useful for mutational analysis of many genes in the *B. cepacia* complex. A system for creating markerless mutations is necessary in order to create multiple deletions in one strain; otherwise the limited number of useful antibiotic resistance markers precludes such studies in the *B. cepacia* complex. This Flp/FRT system is ideally suited for analysis of multidrug resistance since the use of antibiotic resistance markers could mask a drug sensitivity phenotype.

Chapter 5: Literature Cited

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Chapter 6: Appendix

6. APPENDIX: Contribution of p6148 to antibiotic resistance in enteropathogenic *E. coli*

6.1 Introduction

Clinical isolates of *E. coli* are known to contain multiple plasmids. Many of these are small, cryptic plasmids (SCPs) that are maintained despite the fact that they carry very little genetic information (Burian *et al.*, 1997). Some of these small, cryptic plasmids carry antibiotic resistance genes. For example, sulphonamide resistance in *E. coli* usually stems from the acquisition of *sul1* or *sul2* genes, encoding alternate forms of dihydropteroate synthase that are not inhibited by the drug. The *sulI* gene is almost always found on large conjugative plasmids within type I integrons (Radstrom *et al.*, 1991). The *sul2* gene, however, is found on small non-conjugative plasmids in association with streptomycin resistance genes *strA* and *strB* (Radstrom *et al.*, 1991). Among other organisms, the *strA strB sul2* gene cluster can be found on plasmids of clinical *E.coli* and *Shigella* spp. isolates and in large conjugative elements of clinical *Vibrio cholerae* isolates (Enne *et al.*, 2001; Hartman *et al.*, 2003; Hochhut *et al.*, 2001; Ojo *et al.*, 2003). The presence of *sul2*-containing plasmids persists despite a significant decrease in sulphonamide use during the 1990s (Enne *et al.*, 2001). One study suggests that maintenance of *sul2*-plasmids is due to a slight but consistent growth-enhancing effect (Enne *et al.*, 2004).

Two small, cryptic plasmids of unknown function, p6148 and p5218 with sizes of 6.2 kb and 5.0 kb respectively, were isolated from enteropathogenic *E. coli* E2348/69. Sequencing of p6148 revealed the presence of *strA*, *strB*, and *sul2* resistance determinants as shown in Figure 6.1 (J.J. Dennis, personal communication). The objectives of this

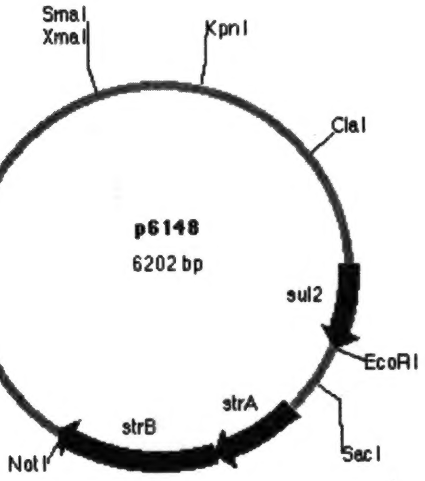
Sma I
Xma I
Kpn I
Cla I
p6148
6202 bp
sul2
EcoR I
Sac I
strA
strB
Not I

Figure 6.1 Physical map of antibiotic resistance genes carried on p6148, a 6.2 kb plasmid from EPEC E2348/69.

study were to examine the role of p6148 in streptomycin and sulphonamide resistance and to determine if the presence of p6148 confers a growth advantage.

6.2 Materials and Methods

6.2.1 Bacterial strains and plasmids

E. coli strains used in this study are listed in Table 6.1. Cloning vectors and recombinant plasmids are given, along with their relevant characteristics, in Table 6.1.

6.2.2 Growth Conditions

Bacterial strains were routinely grown in LB medium or M63 minimal medium (Sambrook *et al.*, 1989) at 37°C with shaking at 250 rpm. When necessary, the following concentrations of antibiotics were used: 100 µg/mL ampicillin, 50 µg/mL kanamycin, 30 µg/mL streptomycin, and 25 µg/mL tetracycline.

6.2.3 Transformation of E. coli JPN15

Cultures of *E. coli* JPN15 were grown in LB medium to an OD_{600} of approximately 0.8. The culture was then divided into 25 mL portions which were kept on ice for 20 minutes then centrifuged for 15 minutes at 4°C. The resulting pellets were washed three times with ice cold sterile water and then resuspended in 100 µL cold 10% glycerol. Cells were kept on ice for immediate use or stored at -80°C. For electroporation, cells were mixed with an appropriate volume of plasmid DNA, transferred into chilled 0.1 cm cuvettes and electroporated using the BIO-RAD (Hercules, CA) MicroPulser setting Ec1 (1.8 kV). After electroporation, cells were immediately

Table 6.1 Bacterial strains and plasmids

E. coli **Strain**	**Relevant Characteristics**	**Reference or Source**
DH5α	F$^-$ Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
E2348/69	Prototypic EPEC isolate (O127 H6)	(Levine *et al.*, 1985)
JPN15	Plasmid-cured derivative of E2348/69, lacking p6148 and the EPEC adherence factor (EAF) plasmid.	(Levine *et al.*, 1985)

Plasmid	**Relevant Characteristics**	**Reference or Source**
p34S-Km3	Source of kanamycin resistance cassette	(Dennis and Zylstra, 1998)
p6148	6.2 kb plasmid from E2348/69 containing *strAB* and *sul2* antibiotic resistance genes	J.J. Dennis, personal communication
p6148-Km (*Kpn*I)	p6148 with the kanamycin resistance cassette from p34S-Km3 inserted into the *Kpn*I site	This study
p6148-Km (*Sac*I)	p6148 with the kanamycin resistance cassette from p34S-Km3 inserted into the *Sac*I site	J.J. Dennis, personal communication
p6148-pUC19 (*Sac*I)	p6148 with pUC19 iserted into the *Sac*I site	J.J. Dennis, personal communication
pLG339	Low copy number cloning vector conferring kanamycin resistance and tetracycline resistance.	(Stoker *et al.*, 1982)
pUC19	High copy number cloning vector conferring ampicillin resistance.	Invitrogen

resuspended in 500 µL SOC, a nutrient rich medium (Sambrook *et al.*, 1989) and incubated for 1 hour at 37°C. Following incubation, cells were plated in 100 µL volumes on LB agar containing selective antibiotics.

6.2.4 DNA analysis

All DNA isolation and general molecular biology techniques were performed as described in Section 2.3.

6.2.5 Overexpression of the sul2 gene

The *sul2* gene was amplified by PCR using p6148 plasmid DNA as template and a 60°C annealing temperature according to the standard PCR reaction described in Section 2.3. The primers *sul*1.L AAGGATCCAAACTCGTCGTTATG and *sul*1.R AAGGATCCAGTCAGAATGCGATT contained engineered *Bam*HI restriction endonuclease sites (underlined) that were used for restriction digestion and ligation of the purified PCR product into the cloning vectors pUC19 and pLG339.

Primers *sul*2.L AAGGATCC**AGGAG**TCGTTATGCATTCGGT and *sul*2.R ACGGATCCAGTTTTCTGATGAAGCG were used to amplify *sul2* from p6148 plasmid DNA, while adding an engineered Shine Dalgarno sequence (bold) that was closer to the *E. coli* consensus. *Bam*HI sites (underlined) were used to clone the resulting PCR product into pLG339.

6.2.6 Minimum inhibitory concentration (MIC) assay

In general, MIC assays were performed as described in Section 2.5. *E. coli* cultures were grown in LB at 37°C to a final OD_{600} of 1.2. Then, 5 µL/well of this culture was used to inoculate 96 well plates containing consecutive two-fold dilutions of an antibiotic stock solution. When a narrower range of antibiotic concentrations was desired, a difference of 250 µg/mL between consecutive wells was used. Following inoculation, the 96 well plates were incubated at 37°C overnight with shaking at 250 rpm. The minimum inhibitory concentration was determined as the lowest concentration that completely inhibited growth, as indicated by OD_{600} measurements.

6.2.7 Growth curves

A single colony of *E. coli* was used to inoculate 5 mL of M63 minimal medium and grown overnight at 37°C. The resulting overnight culture was used to inoculate M63 minimal medium to a final OD_{600} of 0.02. Cultures were incubated at 37°C with shaking at 250 rpm, with OD_{600} readings taken every hour until the culture reached stationary phase.

6.3 Results and Discussion

6.3.1 Effect of p6148 on E. coli streptomycin resistance

The p6148 plasmid contains *strA* and *strB* genes which encode a streptomycin-inactivating phosphotransferase (J.J. Dennis, personal communication). Therefore, this plasmid was expected to confer resistance to streptomycin. To determine if this was the case, the p6148 plasmid from resistant EPEC strain E2348/69 was introduced into *E. coli* JPN15, an isogenic strain that lacks both p6148 and the EPEC adherence factor (EAF) plasmid. Minimum inhibitory concentration assays were performed (Table 6.2). The concentration of streptomycin needed to completely inhibit growth of E2348/69 is approximately 100 times greater than the MIC for JPN15. When p6148 was introduced into JPN15, the streptomycin resistance levels increased to the level of E2348/69 resistance. This result demonstrates that p6148 does confer resistance to streptomycin, and could account for the level of resistance observed in E2348/69.

To determine if the *strA* and *strB* genes were involved in the observed streptomycin resistance, p6148 was interrupted with a kanamycin resistance cassette (p6148-Km(*Sac*I)) or pUC19 plasmid (p6148-pUC19) inserted into the *Sac*I restriction site in the putative *strAB* promoter. The resulting constructs were used to transform JPN15. The MIC of streptomycin on JPN15 carrying p6148-Km(*Sac*I) was identical to that of JPN15. However, when p6148-pUC19 was introduced into JPN15, streptomycin resistance levels were partially restored, approximately 4.5 times that of JPN15 alone (Table 6.2). The above results suggest that the *strA* and *strB* genes are responsible for the streptomycin resistance conferred by p6148. The increased MIC value when p6148-pUC19 is present indicates that some expression of *strA* and *strB* is being driven from a pUC19 promoter. This could be confirmed by performing RT-PCR on the *str* genes.

6.3.2 Effect of p6148 on E. coli sulphonamide resistance

Along with the streptomycin resistance genes, p6148 also contains a *sul2* gene, encoding a sulphonamide resistant form of dihydropteroate synthase (J.J. Dennis, personal communication). To examine the importance of p6148 in EPEC sulphonamide

Table 6.2 The impact of p6148 on streptomycin resistance in *E. coli* JPN15. The values below are the average of three experiments comparing the minimum inhibitory concentration of streptomycin on *E. coli* E2348/69 (the source of p6148), JPN15 (lacks p6148), a pUC19 vector control, and JPN15 that has been transformed with p6148 or interrupted versions of p6148. The plasmids p6148-Km and p6148-pUC19 have a kanamycin resistance cassette or pUC19 inserted into the putative *strAB* promoter.

Strain	**MIC of Streptomycin (µg/mL)**
E2348/69	1000
JPN15	7.8
JPN15 + pUC19	10.4
JPN15 + p6148	1000
JPN15 + p6148-Km (*Sac*I)	7.8
JPN15 + p6148-pUC19 (*Sac*I)	41.6

resistance, minimum inhibitory concentration assays were performed (Table 6.3). The p6148-lacking strain JPN15 was equally resistant to sulphonamides as was EPEC strain E2348/69. The MICs of sulfisoxazole and sulfamethoxazole were 1000 μg/mL, greater than the NCCLS resistance breakpoint of ≥512 μg/mL (Enne *et al.*, 2001). The introduction of p6148 or p6148-Km(*Kpn*I) had no impact on the MIC of either sulphonamide antibiotic, sulfisoxazole or sulfamethoxazole.

To ensure that a small change in resistance was not being missed, MIC assays were repeated using a much narrower range of sulphonamide concentrations (Table 6.4). Again, no differences in the MICs of sulfisoxazole or sulfamethoxale were observed between JPN15 and the p6148-bearing strains. The above results indicate that p6148, and the *sul2* gene, do not contribute to sulphonamide resistance.

The lack of increase in sulphonamide resistance in the presence of p6148 could be the result of a weak or dysfunctional *sul*2 promoter. To address this possibility, the *sul2* gene from p6148 was cloned in both orientations into high copy number plasmid pUC19, placing it under the control of the strong *lac* promoter. The construct was then introduced into *E. coli* DH5α. MIC experiments were performed to determine if the *sul2* gene was capable of conferring sulphonamide resistance (Table 6.5). Rather than increasing resistance, the presence of *sul2* in both orientations actually decreased the MIC of sulphonamides as compared to the wild-type and vector control strains. However, the cells carrying pUC19-*sul2* grew much slower than DH5α with pUC19 alone (data not shown). Thus, it is likely that the decreased resistance is due to a toxic effect rather than an actual sensitivity to sulphonamides. Since the toxic effect was seen even when the

Table 6.3 The impact of p6148 on sulphonamide resistance in *E. coli* JPN15. The values below are the average of three experiments comparing the minimum inhibitory concentration of sulphonamides on *E. coli* E2348/69 (the source of p6148), JPN15 (lacks p6148), and JPN15 that has been transformed with p6148 or p6148 marked with a kanamycin resistance gene.

	MICs (µg/mL)	
Strain	**Sulfisoxazole**	**Sulfamethoxazole**
E2348/69	1000	1000
JPN15	1000	833.3
JPN15 + p6148	833.3	833.3
JPN15 + p6148-Km(*Kpn*I)	833.3	666.7

Table 6.4 The impact of p6148 on sulphonamide resistance in *E. coli* JPN15 measured over a narrow concentration range. The minimum inhibitory concentration of sulphonamides on *E. coli* E2348/69 (the source of p6148), JPN15 (lacks p6148), and JPN15 that has been transformed with p6148 or p6148 marked with a kanamycin resistance gene was determined. A narrow range of sulphonamide concentrations was used, with each consecutive well decreasing by 250 µg/mL. Values are an average of three replicates.

	MICs (µg/mL)	
Strain	**Sulfisoxazole**	**Sulfamethoxazole**
E2348/69	500	500
JPN15	583.3	500
JPN15 + p6148	500	500
JPN15 + p6148-Km(*Kpn*I)	500	500

Table 6.5 Overexpression of the p6148 *sul2* gene from high copy number plasmid pUC19. The *sul2* gene was cloned into the *Bam*HI site of pUC19 and transformed into *E. coli* DH5α. The impact on sulphonamide resistance was determined by comparing the minimum inhibitory concentration of sulfisoxazole and sulfamethoxazole on DH5α, a vector control, and DH5α carrying the *sul2* gene cloned into pUC19 in the same (L-R) or opposite (R-L) orientation as the promoter. Values below are the average of two replicates. Although multiple *sul2* clones were tested, only one representative of each orientation is shown below. However, MIC values were similar for all clones.

	MICs (µg/mL)	
Strain	**Sulfisoxazole**	**Sulfamethoxazole**
DH5α	187.5	312.5
DH5α + pUC19	187.5	187.5
DH5α + pUC19-*sul2* (L-R)	23.4	39.1
DH5α + pUC19-*sul2* (R-L)	31.3	31.3

sul2 gene was cloned in the opposite orientation as the *lac* promoter, there is likely some expression from another pUC19 promoter.

It appeared that the high level of *sul2* expressed from pUC19 was in some way toxic to the cells. To address this concern, we cloned *sul2* into the low copy number plasmid pLG339 and repeated the MIC experiment as described above. Although the growth rate of the *sul2*-bearing cells improved (data not shown), no meaningful increase in sulphonamide resistance was observed (Table 6.6).

Another possible explanation for the lack of change in sulphonamide resistance is that a defective Shine Dalgarno sequence could interfere with translation of *sul2* mRNA. Oligonucleotide primers containing an engineered Shine Dalgarno consensus sequence were used to amplify and clone the *sul2* gene into pLG339. The MICs of sulfisoxazole and sulfamethoxazole were determined for wild-type DH5α, the vector control, and DH5α containing pLG339-*sul2* with the modified Shine Dalgarno sequence (Table 6.7). However, the *sul2* gene was still unable to provide resistance to either sulphonamide antibiotic. The results of *sul2* overexpression experiments demonstrate that neither the promoter nor ribosome binding site is responsible for the lack of sulphonamide resistance. Rather it appears that the *sul2* gene itself is not functional, possibly due to an inactivating mutation.

This result was surprising given the high degree of conservation of *strA strB sul2* gene clusters. For example, *sul2* genes found on several *E. coli* small multicopy plasmids (Enne *et al.*, 2004; Scholz *et al.*, 1989; van Treeck *et al.*, 1981) and on a large, conjugative element (SXT constin) in *Vibrio cholerae* are identical (Hochhut *et al.*, 2001). Isolation of subclones from a *V. cholerae* cosmid library demonstrated that the

Table 6.6 Overexpression of the p6148 *sul2* gene from low copy number plasmid pLG339. The *sul2* gene was cloned into the *Bam*HI site of pLG339 and transformed into *E. coli* DH5α. The impact on sulphonamide resistance was determined by comparing the minimum inhibitory concentration of sulfisoxazole and sulfamethoxazole on DH5α, a vector control, and DH5α carrying the *sul2* gene cloned into pLG339 in the same (L-R) or opposite (R-L) orientation as the promoter. Values below are the average of three replicates. Although multiple *sul2* clones were tested, only one representative of each orientation is shown below. However, MIC values were similar for all clones.

	MICs (µg/mL)	
Strain	**Sulfisoxazole**	**Sulfamethoxazole**
DH5α	250	250
DH5α + pLG339	250	250
DH5α + pLG339-*sul2* (L-R)	333.3	250
DH5α + pLG339-*sul2* (R-L)	583.3	416.7

Table 6.7 Overexpression of the p6148 *sul2* with a modified ribosome binding site from low copy number plasmid pLG339. The *sul2* gene was amplified using primers with an engineered ribosome binding site, cloned into the *Bam*HI site of pLG339, and transformed into *E. coli* DH5α. The impact on sulphonamide resistance was determined by comparing the minimum inhibitory concentration of sulfisoxazole and sulfamethoxazole on DH5α, a vector control, and DH5α carrying the *sul2* gene cloned into pLG339 in the same (L-R) or opposite (R-L) orientation as the promoter. Values below are the average of three replicates. Although multiple *sul2* clones were tested, only one representative of each orientation is shown below. However, MIC values were similar for all clones.

	MICs (μg/mL)	
Strain	**Sulfisoxazole**	**Sulfamethoxazole**
DH5α	250	250
DH5α + pLG339	250	250
DH5α + pLG339-*sul2* (L-R)	250	250
DH5α + pLG339-*sul2* (R-L)	250	416.7

sul2 gene from this organism is able to confer sulphonamide resistance (Hochhut *et al.*, 2001). A comparison of the *sul2* gene sequences from p6148 and the previously characterized elements was needed. Pairwise BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) found that p6148 and p9123 (accession number AY360321) are 99% identical at the DNA level. Their respective *sul2* sequences were even more similar, showing 100% identity. However, one striking difference was observed. The putative p6148 *sul2* ORF, starting at position 429 in Figure 6.2, is only 434 bp long, much shorter than the 816 bp functional *sul2* sequence from p9123 and SXT^{MO10} (accession number AY034138).

A likely explanation for this difference would be the presence of a premature stop codon in the p6148 *sul2* sequence resulting in a partial ORF of only 434 bp. To assess this possibility, a multiple alignment (Gene Tool 2.0, BioTools, Edmonton) of the *sul2* ORFS from p6148, p9123 and SXT^{MO10} was performed (Figure 6.2). As expected, the p6148 *sul2* sequence was almost identical to the ORFs from p9123 and SXT^{MO10}. However, the alignment shows three frameshift mutations (a 1 bp deletion and two 1 bp insertions) and premature stop codons in the p6148 *sul2* region. As a result, the partial *sul2* ORF from p6148 is much smaller than those from p9123 and SXT^{MO10}. This finding explains why addition of p6148 and overexpression of the partial *sul2* ORF did not increase sulphonamide resistance in *E. coli* strains.

6.3.3 Effect of p6148 on the growth rate of E. coli

In 2004, Enne *et al*. reported that the presence of the small plasmid p9123, which carries the *strA strB sul2* gene cluster provided a 3% decrease in logarithmic phase

Figure 6.2 Alignment of the *sul2* sequences and promoter regions from p6148, p9123, and SXT^{M010}. The *sul2* sequence from *E. coli* E2348/69 (p6148) was compared to the characterized *sul2* genes from clinical *E. coli* isolate 91-23 (p9123) and *V. cholerae* (SXT^{M010}). Alignment of these sequences reveals a 1 bp deletion and two 1 bp insertions (dark gray boxes) in the p6148 *sul2* region. These mutations shift the reading frame and create stop codons (* and light gray boxes). As a result, the putative p6148 *sul2* ORF begins with the start codon at postion 429 (underlined).

```
                                                                    M  N
p6148  ATCCGTATGAGACTCATGCTCGATTATTATTATTATAGAAGCCCCCATGAATA 53
                                                                    M  N
p9123  ATCCGTATGAGACTCATGCTCGATTATTATTATTATAGAAGCCCCCATGAATA 53
                                                                    M  N
SXT^M010 ATCCGTATGAGACTCATGCTCGATTATTATTATTATAGAAGCCCCCATGAATA 53

       K  S  L  I  I  F  G  I  V  N  I  T  S  D  S  F  S  D
p6148  AATCGCTCATCATTTTCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGAT 106
       K  S  L  I  I  F  G  I  V  N  I  T  S  D  S  F  S  D
p9123  AATCGCTCATCATTTTCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGAT 106
       K  S  L  I  I  F  G  I  V  N  I  T  S  D  S  F  S  D
SXT^M010 AATCGCTCATCATTTTCGGCATCGTCAACATAACCTCGGACAGTTTCTCCGAT 106

        G  G  R  I  W  R  Q  T  Q  P  L  R  R  R  V  S  *
p6148  GGAGGCC█GTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGCGTAAGCTGAT 158
        G  G  R  Y  L  A  P  D  A  A  I  A  Q  A  R  K  L  M
p9123  GGAGGCC█GTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGCGTAAGCTGAT 159
        G  G  R  Y  L  A  P  D  A  A  I  A  Q  A  R  K  L  M
SXT^M010 GGAGGCC█GTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGCGTAAGCTGAT 159

       W  P  R  G  P  E  C  D  R  P  R  S  G  I  Q  Q  S  R
p6148  GGCCGAGGGGG█CAG█ATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGA 211
         A  E  G  A  D  V  I  D  L  G  P  A  S  S  N  P  D
p9123  GGCCGAGGGGG█CAG█ATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGA 210
         A  E  G  A  D  V  I  D  L  G  P  A  S  S  N  P  D
SXT^M010 GGCCGAGGGGG█CAG█ATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGA 210

        R  R  A  C  F  V  R  H  R  N  R  A  Y  R  A  G  A  G
p6148  CGCCGCGCCTGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGG 264
         A  A  P  V  S  S  D  T  E  I  A  R  I  A  P  V  L
p9123  CGCCGCGCCTGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGG 263
         A  A  P  V  S  S  D  T  E  I  A  R  I  A  P  V  L
SXT^M010 CGCCGCGCCTGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGG 263

         R  A  Q  G  R  W  H  S  R  L  A  R  Q  L  S  T  R
p6148  ACGCGCTCAAGGCAGATGGCATTCCCGTCTCGCTCGACAGTTATCAACCCGCG 317
        D  A  L  K  A  D  G  I  P  V  S  L  D  S  Y  Q  P  A
p9123  ACGCGCTCAAGGCAGATGGCATTCCCGTCTCGCTCGACAGTTATCAACCCGCG 316
        D  A  L  K  A  D  G  I  P  V  S  L  D  S  Y  Q  P  A
SXT^M010 ACGCGCTCAAGGCAGATGGCATTCCCGTCTCGCTCGACAGTTATCAACCCGCG 316

        D  A  S  L  C  L  V  A  W  C  G  L  S  Q  *  Y  S  R
p6148  ACGCAAGCCTATGCCTTGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGG 370
         T  Q  A  Y  A  L  S  R  G  V  A  Y  L  N  D  I  R  G
p9123  ACGCAAGCCTATGCCTTGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGG 369
         T  Q  A  Y  A  L  S  R  G  V  A  Y  L  N  D  I  R  G
SXT^M010 ACGCAAGCCTATGCCTTGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGG 369
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          F  S  R  R  C  V  L  S  A  I  G  E  I  I  C  Q  T  R
p6148    TTTTCCAGACGCTGCGTTCTATCCGCAATTGGCGAAATCATCTGCCAAACTCG 423
           F  P  D  A  A  F  Y  P  Q  L  A  K  S  S  A  K  L
p9123    TTTTCCAGACGCTGCGTTCTATCCGCAATTGGCGAAATCATCTGCCAAACTCG 422
           F  P  D  A  A  F  Y  P  Q  L  A  K  S  S  A  K  L
SXT^M010 TTTTCCAGACGCTGCGTTCTATCCGCAATTGGCGAAATCATCTGCCAAACTCG 422

           R  Y  A  F  G  A  R  R  A  G  R  S  A  R  G  T  R
p6148    TCGTTATGCATTCGGTGCAAGACGGGCAGGCAGATCGGCGCGAGGCACCCGCT 476
         V  V  M  H  S  V  Q  D  G  Q  A  D  R  R  E  A  P  A
p9123    TCGTTATGCATTCGGTGCAAGACGGGCAGGCAGATCGGCGCGAGGCACCCGCT 475
         V  V  M  H  S  V  Q  D  G  Q  A  D  R  R  E  A  P  A
SXT^M010 TCGTTATGCATTCGGTGCAAGACGGGCAGGCAGATCGGCGCGAGGCACCCGCT 475

         W  R  H  H  G  S  H  C  G  V  L  *  R  A  H  R  G  A
p6148    GGCGACATCATGGATCACATTGCGGCGTTCTTTGACGCGCGCATCGCGGCGCT 529
          G  D  I  M  D  H  I  A  A  F  F  D  A  R  I  A  A  L
p9123    GGCGACATCATGGATCACATTGCGGCGTTCTTTGACGCGCGCATCGCGGCGCT 528
          G  D  I  M  D  H  I  A  A  F  F  D  A  R  I  A  A  L
SXT^M010 GGCGACATCATGGATCACATTGCGGCGTTCTTTGACGCGCGCATCGCGGCGCT 528

          D  G  C  R  Y  Q  T  Q  P  P  C  P  *  S  R  H  G  V
p6148    GACGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATCCCGGCATGGGGT 582
           T  G  A  G  I  K  R  N  R  L  V  L  D  P  G  M  G
p9123    GACGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATCCCGGCATGGGGT 581
           T  G  A  G  I  K  R  N  R  L  V  L  D  P  G  M  G
SXT^M010 GACGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATCCCGGCATGGGGT 581

           F  S  G  G  C  S  R  N  L  A  L  G  A  G  A  V  R
p6148    TTTTTCTGGGGGCTGCTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCGAT 635
         F  F  L  G  A  A  P  E  T  S  L  S  V  L  A  R  F  D
p9123    TTTTTCTGGGGGCTGCTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCGAT 634
         F  F  L  G  A  A  P  E  T  S  L  S  V  L  A  R  F  D
SXT^M010 TTTTTCTGGGGGCTGCTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCGAT 634

         *  I  A  A  A  L  R  F  A  G  A  S  V  C  F  A  Q  I
p6148    GAATTGCGGCTGCGCTTCGATTTGCCGGTGCTTCTGTCTGTTTCGCGCAAATC 688
          E  L  R  L  R  F  D  L  P  V  L  L  S  V  S  R  K  S
p9123    GAATTGCGGCTGCGCTTCGATTTGCCGGTGCTTCTGTCTGTTTCGCGCAAATC 687
          E  L  R  L  R  F  D  L  P  V  L  L  S  V  S  R  K  S
SXT^M010 GAATTGCGGCTGCGCTTCGATTTGCCGGTGCTTCTGTCTGTTTCGCGCAAATC 687

          L  S  A  R  A  H  R  P  W  S  G  G  C  R  G  R  D  T
p6148    CTTTCTGCGCGCGCTCACAGGCCGTGGTCCGGGGGATGTCGGGGCCGCGACAC 741
           F  L  R  A  L  T  G  R  G  P  G  D  V  G  A  A  T
p9123    CTTTCTGCGCGCGCTCACAGGCCGTGGTCCGGGGGATGTCGGGGCCGCGACAC 740
           F  L  R  A  L  T  G  R  G  P  G  D  V  G  A  A  T
SXT^M010 CTTTCTGCGCGCGCTCACAGGCCGTGGTCCGGGGGATGTCGGGGCCGCGACAC 740
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           R  C  R  A  C  R  R  R  R  W  S  *  L  H  P  H  T
p6148    TCGCTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACAC 794
         L  A  A  E  L  A  A  A  A  G  G  A  D  F  I  R  T  H
p9123    TCGCTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACAC 793
         L  A  A  E  L  A  A  A  A  G  G  A  D  F  I  R  T  H
SXT^M010 TCGCTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACAC 793

         R  A  A  P  L  A  R  R  A  G  G  I  G  G  A  E  R  N
p6148    GAGCCGCGCCCCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAAC 847
          E  P  R  P  L  R  D  G  L  A  V  L  A  A  L  K  E  T
p9123    GAGCCGCGCCCCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAAC 846
          E  P  R  P  L  R  D  G  L  A  V  L  A  A  L  K  E  T
SXT^M010 GAGCCGCGCCCCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAAC 846

          R  K  N  S  L
p6148    CGCAAGAATTCGTTA                                       862
          A  R  I  R  *
p9123    CGCAAGAATTCGTTA                                       861
          A  R  I  R  *
SXT^M010 CGCAAGAATTCGTTA                                       861
```

generation time as compared to plasmid-free strains. This was observed even when the plasmid was introduced into a naïve host that was not given a chance to adapt to the plasmid. This growth-enhancing effect was not statistically significant, but was observed consistently (Enne *et al.*, 2004). Using similar conditions to those described by Enne *et al.* (2004), we looked for a corresponding growth-enhancing effect from p6148.

Growth curves were performed in M63 minimal media with OD_{600} readings taken every hour (Figure 6.3). Throughout logarithmic phase growth, the p6148-containing E2348/69 grew slightly faster than the other strains. However, there was no difference between the growth rates of JPN15 and JPN15 containing intact p6148. JPN15 carrying the interrupted p6148-Km(*Kpn*I) grew more slowly than the other strains. Based on these results, we conclude that the introduction of p6148 does not provide a growth advantage to *E. coli* JPN15. Although this result contradicts the finding by Enne *et al.* (2004), it is in agreement with the majority of the literature. In general, resistance plasmids impose a fitness cost on their host (Enne *et al.*, 2004; Helling *et al.*, 1981; Johnsen *et al.*, 2002; Lenski *et al.*, 1994; McDermott *et al.*, 1993). Over many generations of co-evolution a plasmid-carrying host may develop compensatory mutations. As a result of these mutations, the plasmid-carrying strain becomes fitter than the plasmid-free strain (Lenski *et al.*, 1994; McDermott *et al.*, 1993). Therefore the introduction of a resistance plasmid into a naïve, plasmid free host, such as JPN15, would not be expected to increase growth rate.

6.4 Conclusions

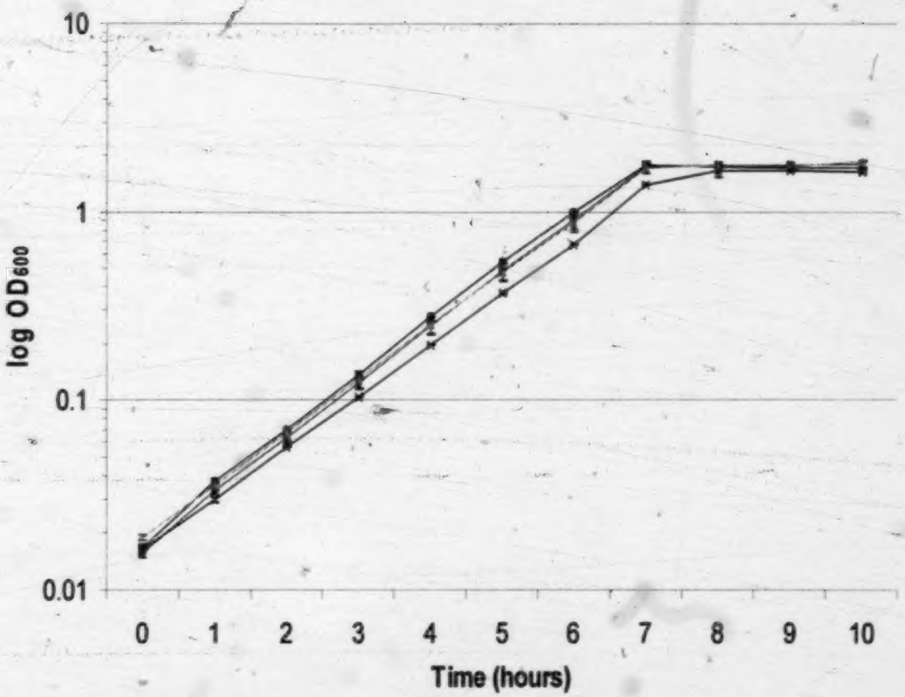

Figure 6.3 Impact of p6148 on the growth of *E. coli*. The p6148 plasmid, intact (triangle) and interrupted with a kanamycin resistance cassette in the *Kpn*I site (X), were transformed into JPN15. Growth in minimal medium was measured for the p6148 containing strains, E2348/69, the source of p6148 (diamond), and JPN15, a strain that lacks p6148 (square). The above graph shows the results of three replicates.

The results of this study have shown conclusively that the p6148 plasmid from *E. coli* E2348/69 confers resistance to streptomycin. Streptomycin resistance was attributed to the presence of *strA* and *strB*, encoding streptomycin phosphotransferase. On the other hand, the *sul2* ORF from p6148 was unable to confer sulphonamide resistance when expressed from p6148 or from cloning vectors pUC19 and pLG339. The lack of sulphonamide resistance was later explained by the presence of a frameshift mutation and premature stop codon upstream of *sul2*. In addition, the reported growth-enhancing effect conferred by p9123, which is 99% identical to p6148, was not observed upon introduction of p6148 into *E. coli* JPN15.